PATENT

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**



Re Application of:
Gabrilovich *et al.*

Serial No.: 09/526,320

Filed: March 15, 2000

For: DENDRITIC CELLS TRANSDUCED WITH A WILD-TYPE SELF GENE ELICIT POTENT ANTITUMOR IMMUNE RESPONSES

Group Art Unit: 1632

Examiner: Wehbe, A.

Atty. Dkt. No.: INRP:074US

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May 14, 2003
Date

Charles P. Landrum

**APPEAL BRIEF**

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## APPEAL BRIEF

Board of Patent Appeals and Interferences
Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

This Appeal Brief is filed in response to the final Office Action mailed on December 12, 2002. The deadline for submission of this Brief is Monday May 19, 2003, by virtue of the date (March 17, 2003) stamped on the return postcard filed with the Notice of Appeal on March 11, 2003 and the fact that May 17, 2003 falls on a Saturday.

The required fees for filing this Appeal Brief ($160.00) is enclosed. Should any other fees be due, or the attached fee be deficient or absent, the Commissioner is authorized to withdraw the appropriate fees from Fulbright & Jaworski Deposit Account No. 50-1212/INRP:074US.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

## STATUS OF THE CLAIMS

Claims 1-11, 15-22, 24, 26-31, 33-37 and 61-135 were pending at the time of the final Office Action. Claims 5-10 and 61-135 are withdrawn from consideration and are canceled in an amendment filed concurrently herewith. Thus, claims 1-4, 11, 15-22, 24, 26-31 and 33-37 were pending at the time of the final Office Action (the "Action"). A copy of the appealed claims is attached as Exhibit A.

## STATUS OF THE AMENDMENTS

An amendment for the purposes of canceling non-elected claims 5-10 and 61-135 is submitted concurrently with this Appeal Brief. A copy of the amendment is attached as Exhibit B.

## STATEMENT OF INTEREST

The real party in interest are the assignees, Introgen Therapeutics, Inc., Austin, TX and Vanderbilt University, Nashville, TN.

## RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

## SUMMARY OF THE INVENTION

The present invention addresses the need for an improved method for treating a human subject having or suspected of having cancer or pre-cancerous disease comprising the steps of (i) identifying a subject having or suspected of having cancer or pre-cancerous disease characterized by alteration or increased expression of a self gene product in at least some of the cancer or pre-cancerous cells in said subject; and (ii) intradermally administering to said subject an expression construct in an adenovirus particle comprising a self gene under the control of a promoter operable in eukaryotic dendritic cells, wherein the dendritic cells are infected by said construct,

whereby said self gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-self gene product response. Specification at least on page 5, lines 26 to 27; page 6, lines 14 to 21 and pages 28 to 31.

The self-gene product can be an oncogene. Specification at least on page 6, line 23 and table 1.

In certain embodiments, the oncogene is selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors. Specification at least on page 6, lines 23 to 26 and table 1.

In another aspect, the tumor suppressor is selected from the group consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1. Further, the tumor suppressor product can be p53. Specification at least on page 6, lines 26 to 30 and 14 to 18.

In certain embodiments, the adenovirus particle can be replication-defective. The replication defect can be a deletion in the E1 region of the virus. In particular embodiments, the deletion maps to the E1B region of the virus. The deletion can encompasses the entire E1B or E1 region of the virus. Specification at least on page 7, lines 19 to 23 and pages 31 to 35.

In certain embodiments, the promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II. In particular embodiments, the promoter is CMV IE. In another aspect, the expression vector further comprises a polyadenylation signal. Specification at least on page 7, lines 25 to 28 and pages 44 to 48.

In certain embodiments, the cancer is selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder. Specification at least on page 8, lines 1 to 7 and page 51.

In particular embodiments, the expression construct is administered via injection. Specification at least on page 8, lines 17 to 18; page 52 and page 64.

In another aspect, the expression construct is administered via injection comprising multiple injections. Specification at least on page 8, lines 17 to 18 and page 52.

Embodiments of the invention include a method wherein the injection is performed local, regional, or distal to a cancer, a pre-cancer or a tumor site. Specification at least on page 8, lines 18 to 22 and page 52.

Embodiments of the invention include a method wherein intradermal administration is via continuous infusion. Specification at least on page 8, lines 22 to 23 and page 65.

In certain embodiments, the immune effector cells are CTLs. Specification at least on page 8, line 15 and page 26.

In another aspect, a method further comprises administering to said subject at least a first cytokine. The method may further comprising administering to the subject a second cytokine, different from the first cytokine. The cytokine may be selected from the group consisting of GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand. In certain embodiments, the cytokine is administered as a gene encoded by the expression construct. Specification at least on page 8, lines 9 to 15.

**ISSUES ON APPEAL**

Do claims 1-4, 11, 15-22, 24, 26-31 and 33-37 satisfy the enablement requirement of 35 U.S.C. 112, first paragraph?

## GROUPING OF THE CLAIMS

For the purposes of this Appeal, the claims stand or fall together.

## SUMMARY OF THE ARGUMENT

The appealed claims satisfy the enablement requirement. In summary, the Examiner has not presented substantial evidence to support the position on appeal that one of ordinary skill would be unable to make and use the invention as claimed.

In contrast, Appellants present evidence in the form of citations to particular portions of the specification and controlling case law that shows that the claimed invention was described in such a manner as to enable one of ordinary skill in the art to make and use the claimed invention.

## ARGUMENT

### A. Substantial Evidence Required to Support the Examiner's Position on Appeal

As an initial matter, Appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act ("APA"), 5 U.S.C. § 706(A), (E), 1994; *see also Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). The Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record pursuant to the APA. *See In re Gartside*, 203 F.3d 1305, 1314-15 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Thus, an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

As explained in the following arguments, the Examiner has not put forth "substantial evidence" that the presently appealed claims are not enabled. In contrast, Appellants have

provided strong evidence in the form of citations to specification and controlling case law that the appealed claims are enabled. The appealed claims are enabled by the specification.

## B. Rejections Under 35 U.S.C. §112, First Paragraph

### 1. Summary of the rejection and the standard for enablement

The Action rejects claims 1-4, 11, 15-22, 24, 26-31 and 33-37 under 35 U.S.C. §112, first paragraph as lacking enablement. In particular, the Examiner contends that the claims lack an enabling disclosure for the treatment of cancer by intradermal injection of an adenovirus encoding any tumor suppressor gene, including p53.

Appellants note that enablement must bear only a *reasonable* relationship to the scope of the claims (*In re Fisher*, 166 U.S.P.Q. 18, 24 (CCPA 1970)). The applicable legal standard does *not* require that all conceivable embodiments encompassed by the claims have been demonstrated to be operable. Applying this correct standard, as explained below, the present claims are fully enabled.

### 2. Claims 1-4, 11, 15-22, 24, 26-31 and 33-37 are Enabled for the Use of Adenovirus Expression Constructs for Transduction of Dendritic Cells

The Examiner alleges, generally, that achieving therapeutic levels of gene expression using currently available vectors is not enabled. In particular, the Examiner cites various articles said to challenge the ability to achieve gene therapy. However, the standard of enablement involves a person who has ordinary skill in and knowledge of the art. No evidence has been provided to indicate that such a person would not be able to employ the teachings of the application, in particular, the use of an adenovirus administered intradermally, with his or her knowledge of the art and treat a human subject having or suspected of having cancer or pre-cancerous disease. Examples need not be presented for every single embodiment of this aspect of the invention. *In re Borkowski*, 164 U.S.P.Q. 624 (CCPA 1970).

An assertion that the disclosure is not commensurate with the scope of the claims must be supported by evidence or reasoning substantiating the doubts advanced. *In re Dinh-Nguyen*, 181 U.S.P.Q. 46 (CCPA 1974). The Examiner has attempted to achieve this by the citation of numerous articles addressing gene therapy in general. As explained below, these citations fall short of what is needed to support the rejection and, furthermore, do not address the methods described in the present specification. Namely, priming of dendritic cells, unlike therapeutic levels of gene expression relied upon in the studies cited, need not rely on high transduction efficiency for therapeutic efficacy due to the function of the dendritic cell in stimulating an immune response (see Kaiserian and Etchart, 1999, page 171, first column last paragraph, (Exhibit C)). Appellants' invention includes priming a dendritic cell using adenoviral vectors for providing an immune response, which is not gene therapy of cancer as generally described in the references cited by the Examiner.

Appellants also note that gene therapy encompasses a wide range of therapeutic methods and generalizations of the entire field do not necessarily apply to all methods of using genetic material as a therapy. For example, the Examiner relies on three references in arguing against the predictability of generating therapeutic levels of gene expression. Moreover, the particular portions of these papers relied upon by the Examiner evince misconceptions about the requirements of §112, first paragraph. For example, Verma *et al.* (Exhibit D) is said to report that "[t]he Achilles heel of gene therapy is gene delivery" and that "most of the approaches suffer from poor efficiency of delivery and transient expression." Verma *et al.* do not address priming of dendritic cells using adenoviral vectors for producing an immune response against cancer. However, all that is required for enablement is *objective* enablement, not any particular level of efficacy. *In re Marzocchi*, 169 UPSQ 370 (CCPA 1971). The Action's reliance on the Verma *et*

*al.* article clearly relates to doubting that current vector systems *are capable of expressing therapeutic proteins in vivo*. However, the number of issued patents that bear on *this issue* indicates otherwise.

Furthermore, Marshall (Exhibit E) and Orkin *et al.* (Exhibit F), are cited as supporting the unpredictability of gene therapy generally and that some problems remain, mainly with regard to therapies that require replacement of defective genes, long term expression of genes and/or the transfection of large number of cells *in vivo*. Marshall and Orkin *et al.* do not address adenoviral priming of dendritic cells for producing an immune response against cancer. However, these references also indicate that, while potentially hampered by limitations, gene therapy does in fact work. Typically, the problems expressed speak to optimization, not bare operability. This is irrelevant to enablement, which is not judged based on commercial applicability or optimization. Thus, despite the Examiner's attempt to "support" the rejection with these citations, it is respectfully submitted that such "evidence" and "reasoning" falls far short of that needed to establish a *prima facie* case of lack of enablement. Furthermore, the shortcomings of such a broad category of therapies are not applicable to the specific invention presently claimed.

In addition, the Examiner references Hurpin *et al.* (Exhibit G) as demonstrating that the route of administration has substantial effects on the ability to generate an immune response. Although Hurpin *et al.* show a difference in ability to generate a CTL response using intradermal administration of a particular poxvirus construct and plasmid vectors, the authors do not address the differences in the vectors such as the use of different promoters or the propensity of the vectors to transfect dendritic cells. Also, the ability of the poxvirus vector was not tested for its ability to protect an animal against a tumor cell challenge. The authors state on page 210, left column, lines 15-18 that "We also saw very little response by the intradermal route which has

been used classically for immunization with the prototypical replication–competent poxvirus, vaccinia." Whether a similar response would be seen when using an adenovirus to treat an animal is only speculation. Thus, the reference does not provide sufficient evidence to demonstrate the inoperability of an adenovirus vector.

The *ex vivo* examples of the specification, demonstrate the propensity of adenovirus for dendritic cells. Furthermore, adenoviral vectors have been shown to elicit humoral and cellular immunity, as described in the specification and in various publications. For example, Gilbert *et al.* (*Vaccine* 15;20(7-8):1039-45, 2002, (Exhibit H)), in particular section 3.1 and figure 1 on page 1041, found that recombinant replication-defective adenovirus expressing the CS gene from *Plasmodium berghei* (Ad-PbCS) induced a strong CD8(+) T cell response after intradermal or muscular injection. Therefore, in view of the working examples in the specification using intravenous, subcutaneous or intraperitoneal injection; the insufficiency of the scientific reason for doubting the enablement of the claimed invention and the evidence provided, Appellants have more than adequately demonstrated the enablement of intradermal administration of an adenovirus in connection with the invention.

Appellants note that there is no requirement or limitation in the claimed invention for specifically targeting a dendritic cell, thus the Examiner's argument regarding dendritic cell targeting is irrelevant to the claimed invention.

### 3. Claims 1-4, 11-31 and 33-37 are Enabled for Methods Comprising Various Self Gene Products

The Examiner, in the Action dated June 26, 2002, rejects claimed methods comprising other tumor suppressor genes. The rejection is based on the general dogma of tumorigenesis in view of Vogelstein *et al.* (Exhibit I) and Restifo *et al.* (Exhibit J). The Examiner concludes that

the specification lacks guidance for the treatment of cells that do not overexpress p53 or do not express p53 at all. The Examiner provides broad generalities that do not rise to a level sufficient to dispute the examples and guidance provided. The argument provided in the Action does not supply *specific* reasons or evidence as to why the use of other self genes would not have a reasonable expectation of success. The art cited supporting the rejection does not provide any evidence that dispute or call into question the extrapolation of the examples provided in the specification to other self genes, particularly tumor suppressor genes.

The scientific reasoning provided previously and currently by the Examiner is not sufficient to establish a *prima facie* case of lack of enablement pertaining to other self genes. Vogelstein *et al.* and Restifo *et al.* do not provide evidence that rebut the exemplary embodiments and guidance provided in the specification. The specification provides a working example for methods of treatment by expression of a tumor suppressor protein by a dendritic cell, as well as guidance for the identification of other self genes. Appellants note that the dendritic cell is a vehicle for presentation of an epitope and any function of a particular polypeptide is irrelevant. The Board is directed to the guidance provided in the specification, at least, on pages 28 to 31, which teaches one of skill in the art how to identify other self genes, including tumor suppressor genes, that are upregulated or have altered expression in cancer cells. This guidance, taken in light of the working examples, teaches one skilled in the art how to identify and use the expression of a self gene by a dendritic cell as a treatment for a cancer involving the over expression or altered expression of a self gene, including a tumor suppressor.

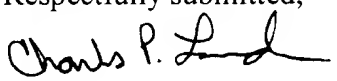
Thus, the heterogeneity of tumor suppressor or oncogene mutations in particular tumors is irrelevant due to the guidance provided in identifying at least one tumor suppressor or oncogene product. Furthermore, the various mechanisms by which tumor cells evade an innate immune

response has no bearing on a stimulated immune response as provided in the examples provided in the instant specification.

**CONCLUSION**

In light of the foregoing, appellants respectfully submit that the claims on appeal should not be rejected under 35 U.S.C. § 112, first paragraph as not being enabled. Reconsideration and withdrawal of the rejection is requested.

Respectfully submitted,



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Date: May 14, 2003

# EXHIBIT A

## EXHIBIT A: PENDING CLAIMS

1. A method for treating a human subject having or suspected of having cancer or pre-cancerous disease comprising the steps of:

   (i) identifying a subject having or suspected of having cancer or pre-cancerous disease characterized by alteration or increased expression of a self gene product in at least some of the cancer or pre-cancerous cells in said subject; and

   (ii) intradermally administering to said subject an expression construct in an adenovirus particle comprising a self gene under the control of a promoter operable in eukaryotic dendritic cells, wherein the dendritic cells are infected by said construct,

whereby said self gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-self gene product response.

2. The method of claim 1, wherein said self-gene product is an oncogene.

3. The method of claim 2, wherein said oncogene is selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors.

4. The method of claim 3, wherein said tumor suppressor is selected from the group consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1.

11. The method of claim 4, wherein said tumor suppressor product is p53.

15. The method of claim 1, wherein said adenovirus particle is replication-defective.

16. The method of claim 15, wherein the replication defect is a deletion in the E1 region of the virus.

17. The method of claim 16, wherein the deletion maps to the E1B region of the virus.

18. The method of claim 17, wherein the deletion encompasses the entire E1B region of the virus.

19. The method of claim 18, wherein the deletion encompasses the entire E1 region of the virus.

20. The method of claim 1, wherein said promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II.

21. The method of claim 20, wherein said promoter is CMV IE.

22. The method of claim 1, wherein said expression vector further comprises a polyadenylation signal.

24. The method of claim 1, wherein said cancer is selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder.

26. The method of claim 1, wherein said expression construct is administered via injection.

27. The method of claim 26, further comprising multiple injections.

28. The method of claim 26, wherein the injection is performed local to a cancer, a pre-cancer or a tumor site.

29. The method of claim 26, wherein the injection is performed regional to a cancer, a pre-cancer or a tumor site.

30. The method of claim 26, wherein the injection is performed distal to a cancer, a pre-cancer or a tumor site.

31. The method of claim 1, wherein intradermal administration is via continuous infusion.

33. The method of claim 1, wherein said immune effector cells are CTLs.

34. The method of claim 1, further comprising administering to said subject at least a first cytokine.

35. The method of claim 34, further comprising administering to said subject a second cytokine, different from said first cytokine.

36. The method of claim 34, wherein said cytokine is selected from the group consisting of GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand.

37. The method of claim 34, wherein said cytokine is administered as a gene encoded by said expression construct.

# EXHIBIT B

**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

*In re* Application of:
Gabrilovich *et al.*

Serial No.: 09/526,320

Filed: March 15, 2000

For: DENDRITIC CELLS TRANSDUCED WITH A WILD-TYPE SELF GENE ELICIT POTENT ANTITUMOR IMMUNE RESPONSES

Group Art Unit: 1632

Examiner: Wehbe, A.

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CERTIFICATE OF MAILING
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I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on the date below:

May 14, 2003
Date

Charles P. Landrum

**AMENDMENT UNDER 37 C.F.R. §1.116**

Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants respectfully submit this amendment to cancel non-elected claims pursuant to 37 C.F.R. 1.144. It is believed that no fees are due, however, should any fees under 37 C.F.R. 1.16 to 1.21 be required for any reason, the Commissioner is authorized to deduct those fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INRP:074US. Please amend the application as indicated below.

## AMENDMENT

**In the Claims:**

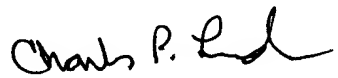
Please cancel claims 5-10 and 61-135 without prejudice or disclaimer.

## REMARKS

The final Office Action dated December 12, 2003, indicated that the claims 5-10 and 61–135, which are non-elected claims, were withdrawn from consideration, but not canceled. Applicants respectfully submit the present amendment to correct status of the non-elected claims.

Should the Examiner have any questions regarding this response, a telephone call to the undersigned is invited.

Respectfully submitted,



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Date: May 14, 2003

## APPENDIX A: PENDING CLAIMS FOLLOWING ENTRY OF THE AMENDMENT

1. A method for treating a human subject having or suspected of having cancer or pre-cancerous disease comprising the steps of:

   (i) identifying a subject having or suspected of having cancer or pre-cancerous disease characterized by alteration or increased expression of a self gene product in at least some of the cancer or pre-cancerous cells in said subject; and

   (ii) intradermally administering to said subject an expression construct in an adenovirus particle comprising a self gene under the control of a promoter operable in eukaryotic dendritic cells, wherein the dendritic cells are infected by said construct,

whereby said self gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-self gene product response.

2. The method of claim 1, wherein said self-gene product is an oncogene.

3. The method of claim 2, wherein said oncogene is selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors.

4. The method of claim 3, wherein said tumor suppressor is selected from the group consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1.

11. The method of claim 4, wherein said tumor suppressor product is p53.

15. The method of claim 1, wherein said adenovirus particle is replication-defective.

16. The method of claim 15, wherein the replication defect is a deletion in the E1 region of the virus.

17. The method of claim 16, wherein the deletion maps to the E1B region of the virus.

18. The method of claim 17, wherein the deletion encompasses the entire E1B region of the virus.

19. The method of claim 18, wherein the deletion encompasses the entire E1 region of the virus.

20. The method of claim 1, wherein said promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II.

21. The method of claim 20, wherein said promoter is CMV IE.

22. The method of claim 1, wherein said expression vector further comprises a polyadenylation signal.

24. The method of claim 1, wherein said cancer is selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder.

26. The method of claim 1, wherein said expression construct is administered via injection.

27. The method of claim 26, further comprising multiple injections.

28. The method of claim 26, wherein the injection is performed local to a cancer, a pre-cancer or a tumor site.

29. The method of claim 26, wherein the injection is performed regional to a cancer, a pre-cancer or a tumor site.

30. The method of claim 26, wherein the injection is performed distal to a cancer, a pre-cancer or a tumor site.

31. The method of claim 1, wherein intradermal administration is via continuous infusion.

33. The method of claim 1, wherein said immune effector cells are CTLs.

34. The method of claim 1, further comprising administering to said subject at least a first cytokine.

35. The method of claim 34, further comprising administering to said subject a second cytokine, different from said first cytokine.

36. The method of claim 34, wherein said cytokine is selected from the group consisting of GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand.

37. The method of claim 34, wherein said cytokine is administered as a gene encoded by said expression construct.

# EXHIBIT C

Eur J Dermatol 1999; 9: 169-76

Dominique KAISERLIAN
Nathalie ETCHART

# Epicutaneous and transcutaneous immunization using DNA or proteins

*(Key words: skin immunization, DNA, dendritic cell, vaccination, Langerhans cells, naked DNA, gene-gun.)*

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Mucocutaneous surfaces are constantly exposed to an array of exogenous antigens including environmental proteins, peptides and low molecular weight and microbial pathogens. These tissues are covered by an epithelium which exerts both the role of a barrier, limiting the penetration of microbes and of hydrophylic antigenic moieties, but at the same time ensures that antigens which penetrate through the epithelium are rapidly captured and transported to draining lymph nodes for initiation of a specific immune response. Epithelial dendritic cells represent the immunocompetent cells responsible for the dynamic uptake and presentation of antigen entering peripheral tissues, and are unique in their efficiency in triggering the immune system and in initiating a primary immune response.

## The skin: a site of immune surveillance

The skin epithelium, the epidermis, is a stratified squamous keratinized epithelium formed by multilayers of keratinocytes (KC), covered by a corneal layer preventing absorption of macromolecules and pathogens. KC are resident cells of non hematopoietic origin, tightened together at the lateral side by desmosomes and at the basal side by hemidesmosomes. The basal KC layer, in contact with the underlying dermis, contains slow dividing stem cells, which differentiate into more superficial and rapidly dividing KC. The skin contains professional antigen-presenting dendritic cells, including Langerhans cells (LC) in the epidermis and dermal dendritic cells (DC) in the dermis. LC, morphologically characterized by their long and slender cytoplasmic processes, from a network of cells protruding their dendrites between KC. Following antigen entry through the skin, LC are rapidly recruited from dermal DC precursors to the epidermis, where they capture and internalize the antigen by endocytosis and start processing native antigen into peptides which are retained in class II vesicles of the endocytic pathway as well as in the endoplasmic reticulum. Antigen capture by LC induces morphological changes and mobilization of the LC, which emigrate through afferent lymphatics (as veiled cells) to the T cell area of draining lymph nodes, where they can be identified as interdigitating DC, forming close contacts with lymph node T cells. At this stage they have acquired expression of costimulatory molecules of the B7 family, upregulated surface expression of MHC class I and class II

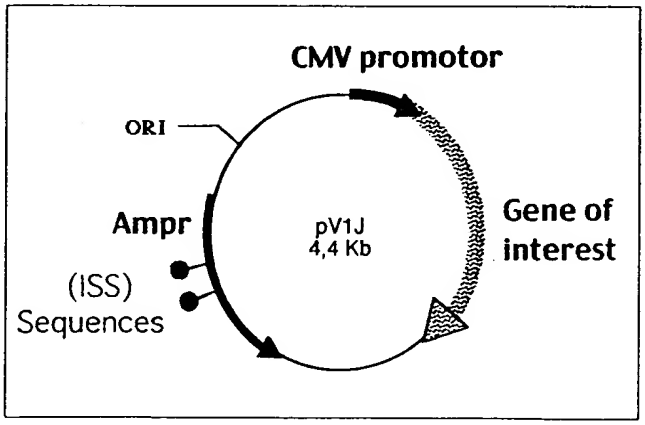


Figure 1. *Schematic diagram of plasmid DNA typically used for DNA immunization.*

molecules bound to peptides, and the ability to secrete high levels of proinflammatory cytokines such as IL-12, IL-1. Thus, interdigitating DC are highly specialized in the activation of naive T cells into antigen-specific T cells.
DC, in contrast to macrophages, are unique in their capacity to prime naive T cells against soluble antigens administered in the absence of an adjuvant. Indeed, although monocytes/macrophages are professional APC capable of the processing and presentation of live or non replicating antigens to activated and memory T cells, they are unable to prime naive T cells efficiently. This is due to the fact that macrophages do not traffic from peripheral tissues to secondary lymphoid organs but are instead attracted at the site of the antigen inflammation, where upon the release of proinflammatory cytokines and chemokines they induce activation of pre-existing antigen specific T and B (memory) cells, recruited from the circulation. Thus, macrophages contribute to antigen presentation during secondary immune responses, but are generally inefficient at priming naive T cells because they do not transport antigen from peripheral sites to T cell areas of secondary lymphoid organs. The migratory property of LC from epithelial tissues to draining lymph nodes together with their unique dynamic changes in antigen processing and presentation functions allow the immune system to recognize foreign antigens encountered in peripheral tissues [reviewed in 1, 2]. In addition, DC were shown to internalize macromolecules by an endocytic pathway which intersects the endogenous cytosolic pathway, allowing class I presentation of exogenous non replicating antigens. Thus, exogenous antigens not synthetized within the DC could elicit cytotoxic T cell responses [3].
During recent years, it has become increasingly clear that manipulation of the immune system for therapeutic (*i.e.* anti-tumoral) or vaccination (anti-infectious) purposes, requires immunization routes allowing efficient antigen uptake by DC. The feasibility of antigen delivery through the skin for the induction of protective immunity against infections has emerged from the field of vaccinology and more recently illustrated by the delivery of sub-unit vaccines. The concept of sub-unit vaccines is based on the idea that harmful complications and potential reversion to virulence, which may occur after vaccination with live attenuated virus or bacteria, thereby limiting their use in immunocompromised individuals, may be overcome by vaccination with antigenic components of the pathogens in the form of recombinant proteins, synthetic peptides, or DNA encoding for the antigen of interest.

## Transcutaneous DNA vaccination

### Basic principles of DNA vaccination

DNA vaccination is based on *in vivo* transfection of host cells with a bacterial DNA plasmid encoding the antigen of interest which can be expressed in host cells, in a way that is similar to that occuring after natural infection.
Plasmid DNA can be delivered in three different forms:
– "naked DNA", consisting of plasmid DNA in solution, is used for immunizations by cutaneous or intramuscular injections;
– plasmid DNA coated onto gold particles, is used for transepidermal immunization by skin bombardment under helium pressure using a gene-gun;
– plasmid DNA encapsulated into inert vectors such as biodegradable microspheres, are suitable for cutaneous injection while live (viral or bacterial) vectors, which can infect skin cells are appropriate for epicutaneous immunization.

*The plasmid*
The bacterial plasmids employed for DNA immunizations generally contain a procaryotic origin of replication and an antibiotic resistance gene suitable for propagation in *E. Coli*. The gene of interest is under the control of a viral promotor and is followed by a mRNA termination/polyadenylation sequence allowing strong expression in mammalian cells *(Fig. 1)*. The plasmid DNA is not infectious and is incapable of replication in eucaryotic cells, is unable to integrate into the host genome and remains episomal in the nucleus of transfected host cells. The encoded antigen is biosynthetized in host cells with native post-translational modifications and protein conformation. Expression of the antigen may persist for prolonged periods of time in cells that are slowly dividing.

*Mode of DNA delivery through the skin*
The skin is an ideal target for DNA immunizations, because it contains numerous and readily accessible bone-marrow-derived LC and dermal DC, specialized in the initiation of the immune response. DNA vaccination through the skin has been performed by coupling recombinant plasmid DNA with < 1 μm gold particles and administration through the skin by bombardement under helium pressure using a gene-gun. Transdermal injection of naked DNA in PBS has also been reported, but is less efficient for priming immune responses, presumably due to the limited uptake by phagocytic cells, compared to DNA coated onto gold beads. Bacterial DNA introduced into viral vectors which can infect a variety of cell types including skin cells, can be administered epicutaneously (see chapter about topical DNA vaccination).

In vivo *priming of a protective immune response*
An increasing number of studies have demonstrated that immunizations with plasmid DNA promote effective immune responses against many bacteria, virus and parasites in rodents [reviewed in 4]. DNA immunization through the skin appears as the most efficient way to prime anti-viral protective CTL responses [5]. Several routes of inoculations of plasmid DNA expressing influenza virus hemagglutinin (subtype H1) have been compared for their ability to induce CTL and antibody responses, and protection against challenge with a mouse adapted influenza virus, expressing the same H1 subtype. These include: (i) intramuscular injection of naked DNA, a route that permits efficient transfection of resident cells, (ii) subcutaneous and intraperitoneal injections, routes that result in less efficient transfection but are frequently used for antigen administration to a test animal, (iii) the epidermis and the upper respiratory tract (nares and trachea) which result in less efficient transfection but deliver DNA to tissues with high levels of local immune surveillance. Mice were immunized twice at 4 week intervals and were challenged on day 10 after the last immunization, by inhalation of the virus into the lung. Apart from the intraperitoneal route, each of these routes gave rise to at least some protection. Intramuscular or intravenous inoculations of naked DNA in saline gave excellent protection but requi-

red large quantities (around 100 μg) of DNA per immunization. Alternatively, mice receiving naked DNA subcutaneously or intradermally had only marginal protection (65-75% survival) and more severe signs of influenza. Transepidermal gene-gun delivery of DNA coated onto gold beads was by far the most efficient method, since protection was achieved with 200-2,000 times less DNA than direct inoculation of DNA in saline. Although expression was transient, and lost in 2-3 days due to normal sloughing of the epidermis, as little as 0.4 μg of DNA was sufficient to achieve 95% survival from lethal influenza challenge. These survivors developed limited-to-no signs of post challenge influenza, in contrast to good survival but more severe influenza in mice receiving naked DNA intranasally.

Similar to intramuscular naked DNA injection, bolistic immunization with DNA coated onto gold beads induces high levels of antibodies to the encoded antigen. However, in different viral systems, the gene-gun induced a predominantly IgG1 (Th2) response with IL-4-producing cells, while intramuscular or intradermal injection of naked DNA induced an IgG2a (Th1) response [6, 7] with expansion of IFN-γ-producing $CD4^+$ T cells and $CD8^+$ CTL. The basis for these divergent responses may reflect the different gene transfer methodologies. Indeed, at least 100 μg of DNA in saline are required for naked DNA injections compared to nanogram quantities of DNA coated onto gold beads for bolistic immunizations. Therefore, injected DNA would provide more bacterial plasmid sequences containing immunostimulatory CpG motifs (ISS), which are known to promote production of proinflammatory cytokines (IL-12 and IFN-α) inducing a Th1-oriented immune response.

That gene-gun vaccination provides protective anti-tumoral CTL responses has been also demonstrated [8]. A single immunization by gene-gun delivery to mice abdominal skin of 1 μg DNA-encoding OVA coated to gold particles, generated OVA-specific CTL activity mediated by class I-restricted $CD8^+$ T cells, as detected in indirect CTL assay, after 7 day restimulation of splenocytes with a syngeneic OVA-transfected lymphoma line (EG7-OVA). Two gene-gun immunizations with a total of 2 μg of DNA-encoding OVA, conferred protection against lethal intradermal injection of OVA-transfected B16 melanoma, while tumors were lethal in all control mice immunized with DNA encoding the irrelevant antigen β-galactosidase. Since the B16 melanoma is non immunogenic in C57BL/6 mice and still remains non immunogenic after OVA transfection, and since OVA endogenously synthetized by OVA-transfected B16 generates the epitope SIINFEKL associated with the class I molecule $K^b$ molecule, these data indicate that DNA immunization induces antigen-specific, CTL-dependent protective tumour immunity.

## Mechanisms of priming of the immune response

*Transfection efficiency* versus *vaccination efficiency*

One of the most striking results of DNA vaccine trials in animal models is that the efficiency of transfection does not necessarily determine the vaccination efficiency. For example, the high ability of rodent muscle to take up and express DNA did not result in better antibody production and protection than intravenous or intranasal inoculations [5]. Thus, it is more likely that transfection of even a limited number of professional APC, such as DC, is preferred over transfection of a large number of resident cells for inducing protective immunity. That DNA inoculation can induce immunological memory is illustrated by the fact that serum titers of specific IgG antibodies are quite low on day 10 after boost, but rapidly increase after challenge, indicating mobilization of memory cells.

In vivo *transfection of DC*

Characterization of the nature of the *in vivo* transfected cells after gene-gun immunization with DNA has been performed using DNA encoded with reporter genes such as the green fluorescence protein (GFP) or β-galactosidase, whose expression in the transfected cells can be easily detected [8]. Gene-gun immunization with DNA results in *in vivo* transfection of both resident KC but also of DC which are highly enriched in the skin. Direct evidence for the uptake of DNA by epidermal DC has, however, not been provided, most likely due to their limited number and their rapid emigration from the skin to draining lymph nodes. However, electron dense 1 μm gold particles have been detected in the cytoplasm of interdigitating lymph node DC, 24 hrs after bolistic immunization [8]. Gene expression by lymph node DC was demonstrated by the green fluorescence of cells with dendritic morphology within T cell areas of the lymph nodes resulting from expression on the green fluorescence protein (GFP) encoded in the DNA used for immunization. Evidence that these *in vivo* transfected DC originated from the skin was further provided by experiments in which skin was painted with the fluorescent hapten rhodamine, immediately before bolistic immunization. Twenty-four hours later, clusters of skin-derived cells (red fluorescence) were evident in the lymph node in the region of afferent lymph flow, and several of these cells were double positive cells (expressing GFP and rhodamine). One cannot formally exclude the possibility that DNA-beads could have travelled through lymphatics to draining lymph nodes and be captured by LN DC or to hepatic lymph and spleen, where they could have been phagocytosed by immature DC, also present at these sites. In this respect, DNA-beads injected subcutaneously can gain access to the cytosol of phagocytic cells. However, because DNA coated onto gold is rapidly solubilized (> 95% within 3 min) in aqueous media, it is unlikely that DNA could survive trafficking to LN uptake by resident DC and endosomal transport. Although solubilized DNA may traffic to lymph nodes and may be captured by DC independently of beads, this could not account for colocalization of beads and DNA expression in the same DC.

*Relative role of keratinocytes (KC) and dendritic cells (DC) to the induction of specific immunity and memory*

When DNA vaccines are administered by gene-gun bombardment of the skin, the majority of the plasmid is taken up by KC [9]. It has been suggested that non migratory cells do not contribute to the development of immunity since primary antibody and CTL responses can be generated despite immediate removal of the site of vaccination [10, 11]. It has been shown that bone-marrow-derived APC contribute to this process [12]. Consistent with this hypothesis, DC in the skin were recently shown to take up DNA-coated beads and migrate within 24 hrs to the draining lymph nodes where primary immunity develops [8]. Recent studies have addressed the role of cells at the site of skin bombardment to the magnitude of the primary res-

ponse and the generation of immunological memory. To this end, transfected skin was periodically removed and transplanted onto naive recipients. Immediate removal of the skin site prevented the outcome of a primary immune response in the vaccinated mice, while both primary immunity and memory could be induced in naive recipients engrafted with skin transferred 0-24 hrs post-vaccination. Thus, skin DC are responsible for both the priming and maintenance of immunological memory. However the magnitude of the primary immune response increased the longer the vaccination site was left in place (for up to 2 weeks). Since transfected KC continue to produce the antigen for up to 2 weeks, this indicates that KC influence the magnitude of the response [13]. Further studies indicate that directly transfected DC exert a predominant role in antigen presentation to $CD8^+$ T cells after gene-gun immunization. Although 24 hrs after immunization the number of lymph node cells directly expressing the transfected DNA are rare (50-100 per individual lymph node), there is a two fold increase in the number of $CD11^+$ DC in the lymph nodes (20-30,000 DC/lymph node). This augmentation is due to gold bombardement and is independent of the presence of plasmid DNA. Using a mutant influenza NP gene which needs the costimulatory B7.2 gene for expression, it was also shown that directly transfected cells were involved in CD8 T cell priming. Only mice immunized with beads co-coated with both plasmids developed NP specific $CD8^+$ T cells, while no priming occured in mice immunized with each plasmid separately. This indicated that antigen presentation was mediated by directly transfected DC *(Fig. 2)* rather than by cross priming due to phagocytosis of transfected cells by DC. Thus, epidermal DNA immunization involves the small number of directly transfected DC migrating to lymph nodes rather than a much larger number of migrating DC that could potientially present the antigen expressed in epidermal cells *via* a cross-priming mechanism [14].

*Adjuvanticity of plasmid DNA: immunostimulatory DNA sequences (ISS)*

DNA vaccines administered in saline are effective in preclinical animal models without the need for adjuvants or delivery systems. Part of this effectiveness may be due to the immunostimulatory effect of the bacterial DNA itself. Specific nucleotide sequences, including unmethylated CpG motifs are immunostimulatory for lymphocytes and are present in naked DNA used for vaccination. For intradermal immunization, incorporation of such stimulatory motif into a plasmid increased both humoral and cellular responses for a weakly antigenic protein β-galactosidase, encoded by the same plasmid or a co-injected plasmid [15]. Furthermore these ISS are responsible for the Th1 response generated after naked DNA injection in the dermis, inasmuch as they suppress IgE production but promote IgG and IFN-γ production. They further initiate the production of IFN-α, IFNβ, IL-12 and IL-18, all of which foster Th1 responses and enhance cell-mediated immunity [16]. KC and dermal DC transfected with the ISS-containing DNA plasmid could produce IL-12 and IFN-α, involved in the induction of a Th1 response against the encoded protein. In this respect *in vitro* studies have shown that CpG-containing oligonucleotides induce activation and maturation of a LC-like fetal skin-derived DC and stimulate production of large amounts of IL-12; injection of CpG nucleotides into the dermis also led to enhanced expression of MHC class II and CD86 (B7.2) molecules by LC in the overlying epidermis with accumulation of IL-12 in a subset of activated LC [17].

It should be noted that the nucleotides flanking CpG motifs also play a role by influencing the nature of the cytokine produced. In addition, the presence within the plasmid of ISS inhibitory sequences may abrogate the immunostimulatory effect of CpG motifs [18].

Thus, the plasmid used for DNA immunizations, itself functions as an adjuvant or an immunomodulator, and altering the nucleotide sequence of the vector may affect the immunogenicity of DNA vaccines. Although ISS are necessary for gene vaccination, they are unnecessary and may be harmful for gene replacement therapy, because they down regulate gene expression as a consequence of induction of IFN-α production by transfected cells.

*CD4 dependency of DNA vaccination*

Intramuscular DNA immunization can prime $CD4^+$ T cells and help antibody responses by class II presentation of exogenous antigens released by *in vivo* transfected cells. Studies have shown that CTL induced by DC pulsed with class I peptides require presentation of class II epitopes and $CD4^+$ T cell activation [19]. In contrast, other studies reported that CTL induced by bolistic immunization using gold beads coated with DNA encoding only a class I-restricted epitope [20] or multiple CTL epitopes appeared to be generated independently of CD4 help [21]. It is possible that antigen presented by directly transfected DC are far more efficient at generating CTL precursors compared to DC pulsed with exogenous class I peptide, not only because of the presence of higher numbers of MHC class I/peptide complexes but also because of continuous antigen synthesis providing repetitive stimulations of naive T cells.

**Increased efficiency of transcutaneous DNA vaccines**

*Co-inoculation of plasmid expressing cytokines*

The efficiency of co-inoculation with plasmids expressing cytokines on the modulation of the immune response to a viral protein encoded in a separate plasmid has been tested by intramuscular route. It was shown that a plasmid vector encoding GM-CSF enhanced the antibody response to a vector encoding rabies G protein, only if both plasmids were injected simultaneously but not if the plasmids were injected separately, even several hours apart. This indicated that either co-transfection of individual cells with both plasmids or close proximity of the APC to GM-CSF secreting cells was crucial, presumably reflecting the localized activity of the cytokine. Co-administration of plasmids encoding GM-CSF resulted in enhanced production of IL-2 and IL-3 but not IL-4, suggesting that Th1 cell response was enhanced. Moreover the presence of a plasmid encoding GMCSF improved the survival of vaccinated mice to intramuscular challenge with rabies virus, from 40% to 80-100% protection [22]. Neutralizing antibodies protected the mice from peripheral challenge by preventing the virus from reaching the central nervous system. Conversely co-immunization with a plasmid encoding mouse IFN-γ, failed to enhance rabies specific T cell response *in vitro* and even decreased specific antibody titers. This most likely reflects the inability of transfected myoblasts to act as APC for stimulation of the immune response, even in circumstances which would favor induction of MHC class II molecules [22]. Epidermal gene-gun immunization with plasmids

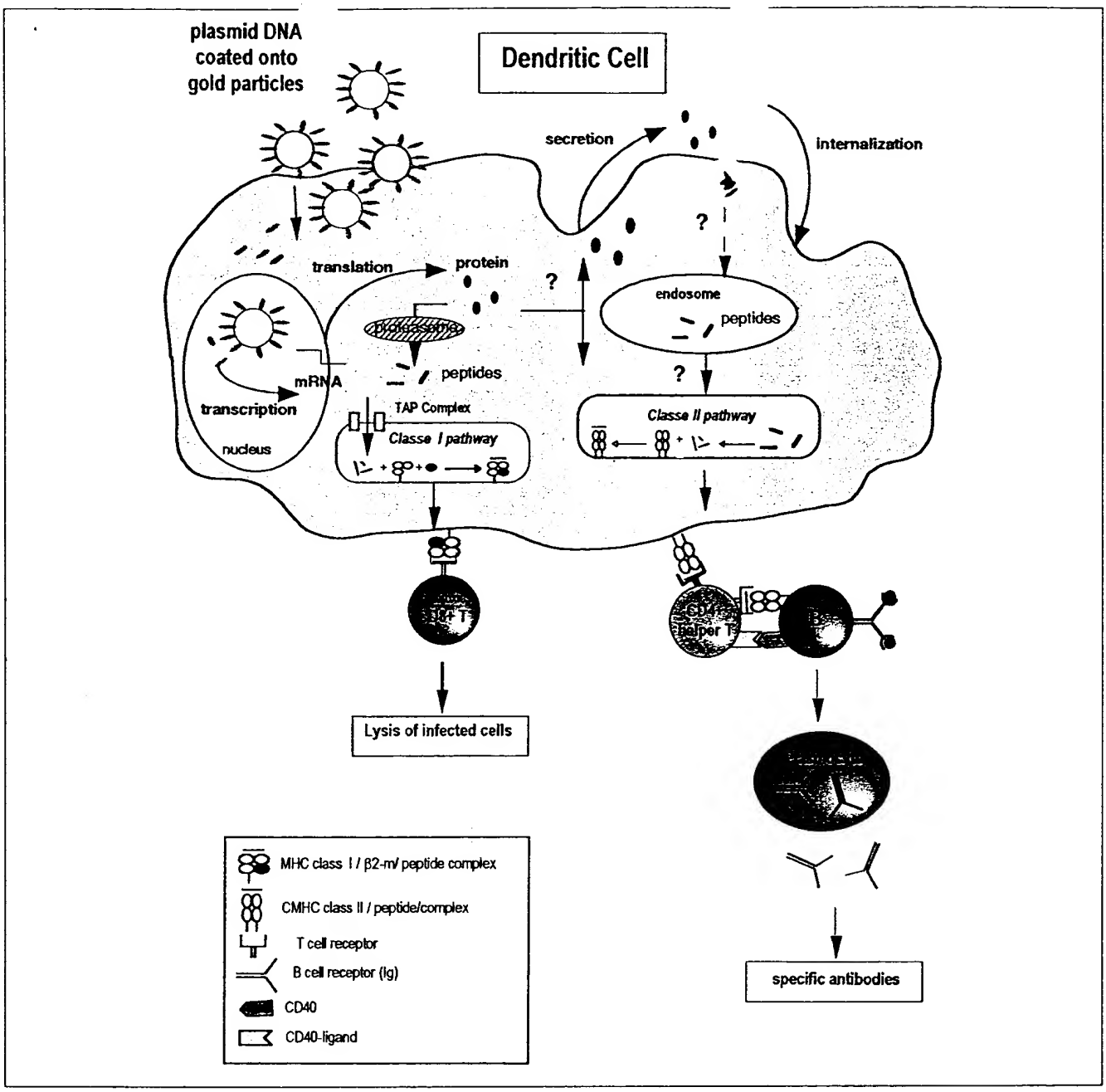


Figure 2. *Hypothetic scheme of antigen presentation after gene-gun immunization with plasmid DNA coated onto gold particles. Plasmid DNA encoding for the antigen of interest and detached from the beads, is transcribed in the nucleus into mRNA coding for the antigen. mRNA exits from the nuclear membrane pores and is translated into protein in the cytosol. The translated protein is then degraded into peptides by a multicatalytic enzymatic complex, the proteasome, and the peptides are transported by the TAP complex into the lumen of the endoplasmic reticulum, for binding to neosynthetized MHC class I molecules (class I pathway). MHC class I/peptide complexes are then transported through exocytic vesicles to the cell surface for presentation to CD8⁺ T cells. The pathway of antigen presentation by MHC class II molecules is not clear and most likely depends on the nature of the encoded protein. Secreted proteins released in the extracellular milieu may be internalized by the endocytic pathway* (i.e. *class II pathway), processed into peptides in endosomal vesicles where then can bind to MHC class II molecules (class II pathway) and presented to CD4⁺ T cells. In contrast the mechanism by which cytosolic proteins are processed and presented by class II molecules is unclear. It is possible that lysis of the APC results in the release of the cytosolic protein antigen outside the cell and internalization through the endocytic pathway.*

encoding IFN-α and IL-12 along with antigen-encoding plasmids are able to enhance CTL activity and shift the normally observed Th2 bias of the immune response towards a Th1 phenotype (LD Falo, manuscript in preparation).

*Targeting DNA to sites of immune induction*

Because the availability of antigen in lymphoid organs is important in generating an immune response, it has been postulated that antigen availability may also be important

in the response to DNA vaccines, because immune responses are stronger when antigen is secreted from DNA-transfected cells. In order to direct expression of the transgene to lymphoid organs, vaccination was performed using DNA encoding antigen-ligand fusion proteins. The model antigen human IgG was targeted to lymph nodes or APC by two ligands, L-selectin or CTLA4-Ig, respectively binding to receptors that are present on endothelial venule cells of lymph nodes and on antigen presenting cells. L-selectin, expressed on the surface of naive lymphocytes and by binding to CD34 on high endothelial venule cells, initiates entry into lymph nodes, CTLA4 on activated T cells binds to B7-expressing APC, which are required to initiate the immune response. Intramuscular injection of DNA encoding each of these fusion proteins dramatically enhanced the humoral response to human IgG (by $10^4$ fold within 2 to 8 weeks) as well as T cell proliferative responses. Targeting of the DNA vaccine to endothelial venules by L-selectin enhanced the Th1 response (IgG2a and IgG2b) that was observed after intramuscular immunization with plasmid encoding Hu IgG alone, while targetting to APC by CTLA4Ig induced a switch towards a Th2 type response as shown by a 7,000 fold increase in IgG1 level [23].

Another approach to increase the efficiency of DNA immunization is targeting DNA to APC. As several types of APC, including DC, phagocytose particulate material in the micron size range, plasmid DNA has been encapsulated in biodegradable polylactide-co-glycolide (PLGA) microspheres. Such microparticles when engulfed by APC *in vitro* showed expression of the encoded protein within 24 hrs and up to 3 days. In addition, microspheres containing 2 µg of plasmid DNA encoding for a single class I epitope of Vesicular Stomatitis Virus, elicited a VSV-specific CTL *in vivo*, when injected subcutaneously. It is interesting to note that the CTL response induced by a single immunization with 2 µg of encapsulated DNA was higher than that induced by two intramuscular injections with 200 µg naked DNA or VSV peptide in complete Freund adjuvant. The data illustrate that the efficiency of DNA encapsulation may be due both to the protective nature of the polymer coating and to the increased uptake of DNA by phagocytic APC [24].

## Epicutaneous immunization with DNA or proteins

### Topical DNA vaccination by utilization of viral vector

Viral vectors such as adenovirus, which has a broad host range and can infect non dividing cells, offer a tool for gene therapy of skin diseases. Adenovirus carrying plasmid DNA encoding β galactosidase, can be delivered topically on to the skin of mice. This is performed by first tape-stripping of the corneal layer, then application of the recombinant virus onto 1 $cm^2$ of skin by an occlusive technique. Transduction of the antigen appears by 2-3 hrs and within 24-48 hrs the antigen is expressed on the entire skin, including all keratinocytes and some follicular cells, with a mild mononuclear cell and neutrophil infiltrate of the epidermis. At day 2, the level of β-galactosidase expression in the skin after topical adenovirus vector is 14 fold higher than that observed after parallel gene-gun immunization with the same plasmid. In addition, topical skin immunization for 2 days with an adenovirus vector expressing TGF-α, results 4 days later in hyperkeratosis and acanthosis demonstrating that the transduced gene is biologically active [25]. This demonstrated that replication defective viral vector can be used for efficient topical skin immunization.

### Epicutaneous immunization with proteins

The skin's great barrier properties limits the penetration of macromolecules greater than 500 Da, thus preventing epicutaneous delivery of the high molecular weight therapeutics as well as non invasive transcutaneous immunization. However, it has been recently shown that transcutaneous immunization can be achieved by simple application of protein antigens mixed with cholera toxin (CT) onto the shaved mouse skin. CT is a member of ADP-ribosylating bacterial exotoxins, widely used experimentally as an adjuvant to enhance immune response to vaccine components, by the oral and the nasal routes. CT binds to asialo-GM1 ganglioside expressed on a variety of epithelial and hematopoietic cell types through its B subunit. It was observed that two epicutaneous applications of 100 µg of CT alone, performed 3 or 8 weeks apart, induced specific serum IgG. Furthermore, CT could act as a skin adjuvant for the common vaccine components diphteria toxoid and tetanus toxoid, by promoting induction of specific antibodies to the vaccines. No sign of redness or swelling of the skin at the site of application were observed up to 72 hrs post-immunization and skin biopsies taken at the site of exposure revealed no sign of inflammation. Thus, transcutaneous immunization with large proteins without physical penetration of the skin by needles can be achieved using CT. In addition, conversely to administration by mucosal routes, CT is non toxic when applied epicutaneously, without skin disruption [26].

Moreover, it was also shown that epicutaneous immunization with CT alone induced anti-CT IgG and IgA antibodies in the serum, lung washes and stool samples and that immunized mice were protected from an intranasal challenge with a lethal dose of CT. Therefore epicutaneous CT administration induced clinically relevant immunity against mucosal toxin challenge [27]. However, although CT has been shown to induce a predominantly Th2-biased immune response, it has been shown that IgE were also induced when CT was used as adjuvant. More careful examination of IgE responses in these studies has not been undertaken. This is, however, of major concern for skin immunization with proteins, particularly because of the potential risk of inducing immediate hypersensitivity reactions.

There is one exception to the theory that proteins cannot normally penetrate through the skin: the skin of patients with atopic dermatitis which exhibits deficient barrier functions [28]. Indeed, eczematous reactions can develop after epicutaneous application of protein allergens on the non lesional skin of atopic dermatitis patients, demonstrating that high mw proteins could penetrate through this type of skin and induce an allergen-specific DTH response [29]. These observations suggested that the skin, like the gut and the lungs, could be a site of sensitization to environmental protein allergens in these patients. In this respect, two independent studies in normal inbred strains of mice have shown that epicutaneous exposure to ovalbumin, in the absence of adjuvant, can sensitize the animals and induce a

dominant Th2-like response with nigh levels of specific IgE. Moreover, repeated immunizations sustained elevated levels of IgE. The protocole of epicutaneous immunization used was performed by applying the immunizing solution on a patch left in place for 3 consecutive days on the shaved back skin and immunization was renewed by application of a freshly prepared patch for another 3 days [30]. Similar observations were reported by another group who further showed that mice exposed to 3 one week 100 µg OVA skin patches, separated from each other by 2 week intervals, not only developed IgE responses, but also developed dermatitis with skin infiltration with $CD3^+$ T cells, eosinophils and neutrophils. RNA for IL-4, IL-5 and IFN-γ were detected in the skin. More strikingly, a single epicutaneous exposure to OVA induced eosinophilia in the bronchoalveolar lavage fluid, and airway hyperresponsiveness to intravenous methacholine [31]. These data suggest that epicutaneous exposure to protein antigens in atopic dermatitis may be involved in the development of allergic asthma. Furthermore, this also raises questions about the feasibility of epicutaneous protein antigen delivery for vaccination purposes.

The Th2-biased immune response following protein application contrasts with the Th1-biased response generated by topical exposure to sensitizing haptens. The explanation for these different types of immune response are not clear. It is possible that the higher number of MHC/peptide complexes generated by hapten immunization as compared to those generated by protein processing, reflects the predominant Th1 response after contact sensitization with haptens compared to the Th2 response generated with proteins. In addition, contact sensitizers are all irritants and induce inflammation while proteins are not inflammatory unless administered with adjuvants. Finally, the nature of the protein itself and the presence of sequences endowed with protease activity dictates the allergenic potential of proteins [32], through as yet unknown mechanisms. The risk of developing a Th2-biased response and allergy may thus also depend on the nature of the protein.

## Prospective use of DNA for the treatment of skin lesions

Insertion and expression of genes in the epidermis may have a variety of therapeutic uses, including the treatment of skin diseases by the expression of cytokines and other biologically active molecules for the treatment of skin lesions. For example, genes encoding IFN-α could be injected to treat skin tumors and viral lesions (Kaposi's sarcoma, basal cell carcinoma, cutaneous squamous cell carcinoma, papilloma). In addition, gene-gun vaccination with plasmid encoding cytokines may be advantageous for the treatment of cancer, rather than intravenous cytokine delivery which is often inefficient and frequently accompanied by systemic cytotoxicity.

It should be emphasized that in contrast to mouse skin, in which injected DNA is expressed not only in the epidermis but also in the dermis and the underlying fat and muscle tissue and is expressed at low levels, DNA injected into the superficial dermis of human or pig skin organ cultures and in human skin grafted onto immunocompromised mice is taken up and expressed in the epidermis [33]. These data further indicate that the pig is an appropriate model for preclinical studies on DNA vaccination and therapeutics.

In addition to skin vaccination, mucocutaneous gene therapy offers new potential approaches for local treatment of various skin lesions. The feasibility of gene-gun delivery of DNA through the oral mucosa or the epidermis has been tested in dogs, which develop spontaneous oral and epidermal tumors. Pilot studies using the reporter gene β-galactosidase inserted into a CMV plasmid showed that direct injection of 20 µg of plasmid into the oral mucosa induced 35 fold higher local expression of β-galactosidase as compared to injection of the same dose in the epidermis. Due to the accelerated turn over of mucosal epithelium, β-galactosidase positive cells were detected in the basal and suprabasal layers as early as 3 hrs after injection, whereas only the most superficial mucosal layers demonstrated β-galactosidase activity at 24 hrs post-injection [34]. It was observed that particle-mediated gene transfer of either β-galactosidase, luciferase, IL-2, IL-6 or GM-CSF cDNA into the oral mucosa and the epidermis of healthy dogs generated effective and localized transgene expression, without signs of toxicity. Other studies confirmed and extended these observations by showing that injection of DNA encoding IL-8 directly into the epidermis is able to recruit neutrophils into the underlying dermis [35]. Thus, the gene-gun approach should be considered for potential clinical applications in cancer immunotherapy.

Nucleic acid inoculation through the skin represents a promising tool in the field of vaccinology and treatment of skin lesions. Plasmid DNA immunization has been used in a number of animal models as a novel strategy to induce both antibodies and CTL responses as well as long term protection against infectious agents or tumor development. Moreover, vaccines consisting of plasmid DNA have several important advantages over alternate approaches such as purified or recombinant proteins and live-attenuated or recombinant viruses. They can be easily constructed for any suitable antigen and produced economically in large quantities with a high degree of purity and stability and thus are appropriate for mass immunization. ■

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# EXHIBIT D

# Gene therapy — promises, problems and prospects

Inder M. Verma and Nikunj Somia

**In principle, gene therapy is simple: putting corrective genetic material into cells alleviates the symptoms of disease. In practice, considerable obstacles have emerged. But, thanks to better delivery systems, there is hope that the technique will succeed.**

In 1990, the first clinical trials for gene-therapy approaches to combat disease were carried out. Conceptually, the technique involves identifying appropriate DNA sequences and cell types, then developing suitable ways in which to get enough of the DNA into these cells. With efficient delivery, the therapeutic prospects range from tackling genetic diseases and slowing the progression of tumours, to fighting viral infections and stopping neurodegenerative diseases. But the problems — such as the lack of efficient delivery systems, lack of sustained expression, and host immune reactions — remain formidable challenges.

Although more than 200 clinical trials are currently underway worldwide, with hundreds of patients enrolled, there is still no single outcome that we can point to as a success story. To explore why this is the case, we will use our own experience and other examples to look at the many technical, logistical and, in some cases, conceptual hurdles that need to be overcome before gene therapy becomes routine practice in medicine.

At present, gene therapy is being contemplated only on somatic (essentially, non-reproductive) cells. Although many somatic tissues can receive therapeutic DNA, the choice of cell usually depends on the nature of the disease. Sometimes a clear definition of the target cell is needed. For example, the gene that is defective in cystic fibrosis has been identified, and clinical trials to deliver DNA as an aerosol into the lung have already begun[1]. Although cystic fibrosis is manifest in this organ, it is still not clear that delivery of a correcting gene by this method will reach the right type of cell. On the other hand, to correct blood-clotting disorders such as haemophilia, all that is needed is a therapeutic level of clotting protein in the plasma[2]. This protein may be supplied by muscle or liver cells, fibroblasts, or even blood cells[3–5]. The choice of tissue in which to express the therapeutic protein will also ultimately depend on considerations such as the efficiency of gene delivery, protein modifications, immunological status, accessibility and economics.

We also need to consider how much of the therapeutic protein should be delivered. In haemophilia B, which is caused by a deficiency of a blood-clotting protein called factor IX, giving patients just 5% of the normal circulating levels of this protein can substantially improve their quality of life[2]. Most people have about 5 μg of factor IX per millilitre of plasma, produced by the $10^{13}$ cells that make up the liver. So we need to deliver a correcting gene to $5 \times 10^{11}$ cells — that is, 5% of liver cells. Alternatively, fewer liver cells would need to be modified if more factor IX could be produced per cell, without being deleterious. In the brain, however, gene transfer to just a few hundred cells could considerably benefit patients with neurological disease. And finally, we can consider the transfer of genes to a handful of stem (or progenitor) cells, which grow and divide to generate millions of progeny. The range in the number of cells that this technology has to cover is vast.

The Achilles heel of gene therapy is gene delivery, and this is the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression. There are two categories of delivery vehicle ('vector'). The first comprises the non-viral vectors, ranging from direct injection of DNA to mixing the DNA with polylysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from poor efficiency of delivery and transient expression of the gene[6]. Although there are reagents that increase the efficiency of delivery, transient expression of the transgene is a conceptual hurdle that needs to be addressed.

Most of the current gene-therapy approaches make use of the second category — viral vectors. Importantly, the viruses used have all been disabled of any pathogenic effects. The use of viruses is a powerful technique, because many of them have evolved a specific machinery to deliver DNA to cells. However, humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses. ▶

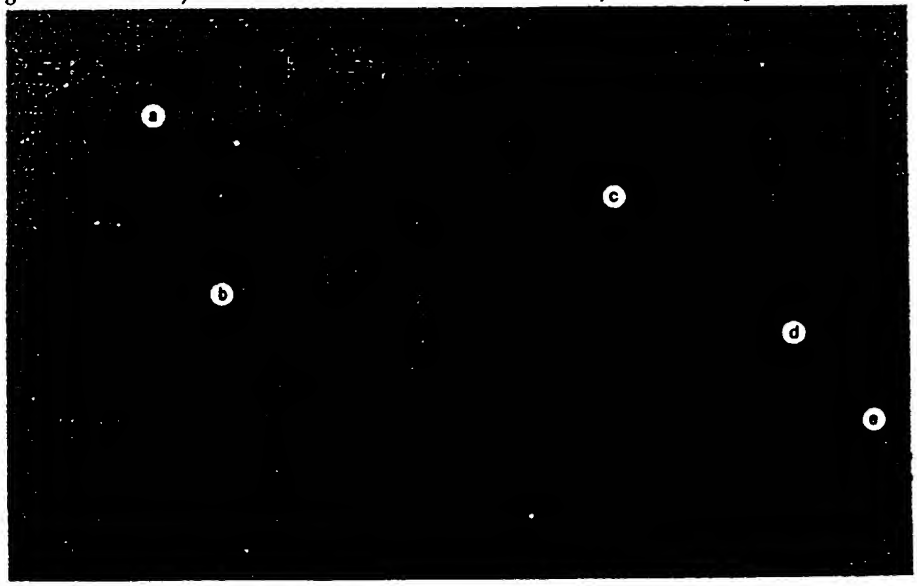



Figure 1 To create the retroviral vectors that are used in gene therapy, the life-cycles of their naturally occurring counterparts are exploited. a, The transgene (in this case, the gene for factor IX) in a vector backbone is put into a packaging cell, which expresses the genes that are required for viral integration (*gag*, *pol* and *env*). b, The transgene is incorporated into the nucleus, where it is transcribed to make vector RNA. This is then packaged into the retroviral vector, which is shed from the packaging cell. c, The vector is delivered to the target cell by infection. The membrane of the viral vector fuses with the target cell, allowing the vector RNA to enter. d, The virally encoded enzyme reverse transcriptase converts the vector RNA into an RNA–DNA hybrid, and then into double-stranded DNA. e, The vector DNA is integrated into the host genome, then the host-cell machinery will transcribe and translate it to make RNA and, in this case, factor IX protein.
LTR, long terminal repeat; ψ, packaging sequence.

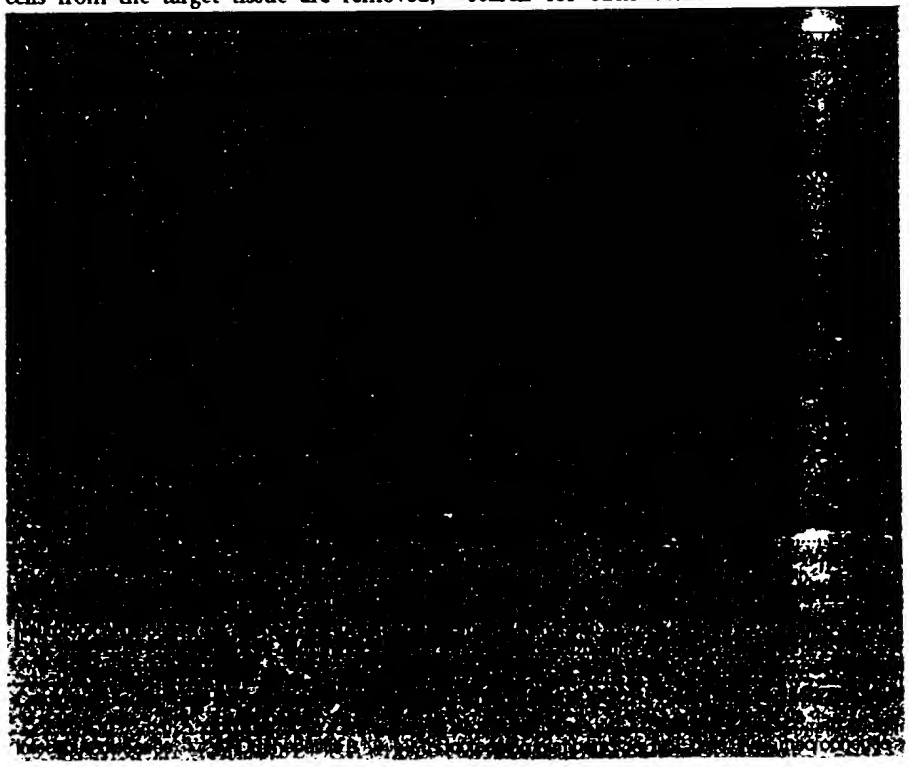
### Retroviral vectors

Retroviruses are a group of viruses whose RNA genome is converted to DNA in the infected cell. The genome comprises three genes termed *gag*, *pol* and *env*, which are flanked by elements called long terminal repeats (LTRs). These are required for integration into the host genome, and they define the beginning and end of the viral genome. The LTRs also serve as enhancer–promoter sequences — that is, they control expression of the viral genes. The final element of the genome, called the packaging sequence ($\psi$), allows the viral RNA to be distinguished from other RNAs in the cell (Fig. 1)[7].

By manipulating the viral genome, viral genes can be replaced with transgenes — such as the gene for factor IX (Table 1). Transcription of the transgene may be under the control of viral LTRs or, alternatively, enhancer–promoter elements can be engineered in with the transgene. The chimaeric genome is then introduced into a packaging cell, which produces all of the viral proteins (such as the products of the *gag*, *pol* and *env* genes), but these have been separated from the LTRs and the packaging sequence. So, only the chimaeric viral genomes are assembled to generate a retroviral vector. The culture medium in which these packaging cells have been grown is then applied to the target cells, resulting in transfer of the transgene. Typically, a million target cells on a culture dish can be infected with one millilitre of the viral soup.

A critical limitation of retroviral vectors is their inability to infect non-dividing cells[8], such as those that make up muscle, brain, lung and liver tissue. So, when possible, the cells from the target tissue are removed, grown *in vitro*, and infected with the recombinant retroviral vector. The target cells producing the foreign protein are then transplanted back into the animal. This procedure has been termed '*ex vivo* gene therapy' and our group has used it to infect mouse primary fibroblasts or myoblasts (connective-tissue and muscle precursors, respectively) with retroviral vectors producing the factor IX protein. But within five to seven days of transplanting the infected cells back into mice, expression of factor IX is shut off[3,5,9]. This transcriptional shut-off has even been observed in mice lacking a functional immune system (nude mice), and it cannot be due to cell loss or gene deletion[5] because the transplanted cells can be recovered.

What is the mechanism of this unexpected but intriguing problem? We do not yet know, but the exceptions may provide some clues. To obtain sustained expression in mouse muscle following the transplantation of infected myoblasts, we used the muscle creatine kinase enhancer–promoter to control transcription of the transgene. Unfortunately, this is a weak promoter, and only low levels of protein were produced. So, we generated a chimaeric vector in which the muscle creatine kinase enhancer was linked to a strong promoter from cytomegalovirus. Using this vector, sustained and high levels of factor IX were expressed when the infected myoblasts were transplanted back into mice. Remarkably, these expression levels remained unchanged for more than two years (the life of the animal). So we can override the 'off switch' and achieve higher levels of expression by using an appropriate enhancer–promoter combination. But the search for such combinations is a case of trial and error for a given type of cell.



Another formidable challenge to the *ex vivo* approach is the efficiency of transplantation of the infected cells. Attempts to repeat the long-term myoblast transplantation in haemophiliac dogs led to only short-term expression, because the infected dog myoblasts could not fuse with the muscle fibres. So perhaps successful animal models will prove inadequate when the same protocols are extended to humans. Moreover, these models are based on inbred animals — the outbred human population, with individual variation, will add yet another degree of complexity. The haematopoietic (blood-producing) system may offer an advantage for *ex vivo* gene therapy because resting stem cells can be stimulated to divide *in vitro* using growth factors and the transplantation technology is well established. But there is still a lack of good enhancer–promoter combinations that allow sustained production of high levels of protein in these cells.

Another problem is the possibility of random integration of vector DNA into the host chromosome. This could lead to activation of oncogenes or inactivation of tumour-suppressor genes. Although the theoretical probability of such an event is quite low, it is of some concern (see section on clinical trials).

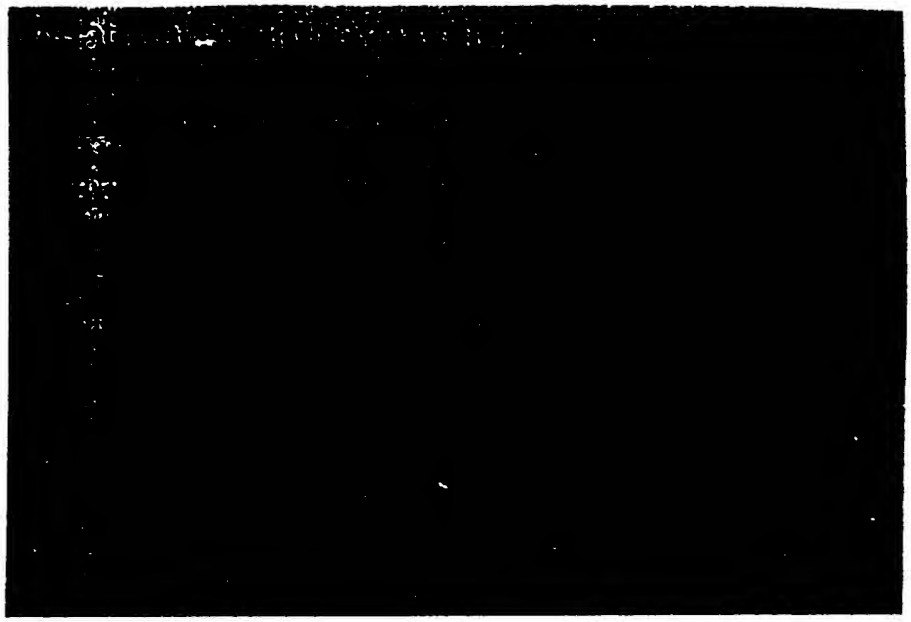
### Lentiviral vectors

Lentiviruses also belong to the retrovirus family, but they can infect both dividing and non-dividing cells[10]. The best-known lentivirus is the human immunodeficiency virus (HIV), which has been disabled and developed as a vector for *in vivo* gene delivery. Like the simple retroviruses, HIV has the three *gag*, *pol* and *env* genes, but it also carries genes for six accessory proteins termed *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*[11].

Using the retrovirus vectors as a model, lentivirus vectors have been made, with the transgene enclosed between the LTRs and a packaging sequence[12]. Some of the accessory proteins can be eliminated without affecting production of the vector or efficiency of infection. The *env* gene product would restrict HIV-based vectors to infecting only cells that express a protein called $CD4^+$ so, in the vectors, this gene is substituted with *env* sequences from other RNA viruses that have a broader infection spectrum (such as glycoprotein from the vesicular stomatitis virus). These vectors can be produced — albeit on a small scale at the moment — at concentrations of $>10^9$ virus particles per ml (ref. 12).

When lentivirus vectors are injected into rodent brain, liver, muscle, eye or pancreatic-islet cells, they give sustained expression for over six months — the longest time tested so far[13,14]. Unlike the prototypical retroviral vectors, the expression is not subject to 'shut off'. Little is known about the possible immune problems associated with lentiviral vectors, but injection of $10^7$ infectious units

does not elicit the cellular immune response at the site of injection. Furthermore, there seems to be no potent antibody response. So, at present, lentiviral vectors seem to offer an excellent opportunity for *in vivo* gene delivery with sustained expression.



**Adenoviral vectors**

The adenoviruses are a family of DNA viruses that can infect both dividing and non-dividing cells, causing benign respiratory-tract infections in humans[11]. Their genomes contain over a dozen genes, and they do not usually integrate into the host DNA. Instead, they are replicated as episomal (extrachromosomal) elements in the nucleus of the host cell. Replication-deficient adenovirus vectors can be generated by replacing the *E1* gene — which is essential for viral replication — with the gene of interest (for example, that for factor IX) and an enhancer–promoter element. The recombinant vectors are then replicated in cells that express the products of the *E1* gene, and they can be generated in very high concentrations ($>10^{11}$–$10^{12}$ adenovirus particles per ml)[15].

Cells infected with recombinant adenovirus can express the therapeutic gene but, because essential genes for viral replication are deleted, the vector should not replicate. These vectors can infect cells *in vivo*, causing them to express very high levels of the transgene. Unfortunately, this expression lasts for only a short time (5–10 days post-infection). In contrast to the retroviral vectors, long-term expression can be achieved if the recombinant adenoviral vectors are introduced into nude mice, or into mice that are given both the adenoviral vector and immunosuppressing agents[16]. This indicates that the immune system is behind the short-term expression that is usually obtained from adenoviral vectors.

The immune reaction is potent, eliciting both the cell-killing 'cellular' response and the antibody-producing 'humoral' response. In the cellular response, virally infected cells are killed by cytotoxic T lymphocytes[16,17]. The humoral response results in the generation of antibodies to adenoviral proteins, and it will prevent any subsequent infection if the animal is given a second injection of the recombinant adenovirus. Unfortunately for gene therapy, most of the human population will probably have antibodies to adenovirus from previous infection with the naturally occurring virus.

It is possible that the target cell contains factors that might trigger the synthesis of adenoviral proteins, leading to an immune response. To try to get around this problem, second-generation adenoviral vectors were developed, in which additional genes that are implicated in viral replication were deleted. These vectors showed longer-term expression, but a decline after 20–40 days was still apparent[18]. This idea has now been taken further with the generation of 'gut-less' vectors — all of the viral genes are deleted, leaving only the elements that define the beginning and the end of the genome, and the viral packaging sequence. The transgenes carried by these viruses were expressed for 84 days[19].

There are considerable immunological problems to be overcome before adenoviral vectors can be used to deliver genes and produce sustained expression. The incoming adenoviral proteins that package DNA can be transported to the cytoplasm where they are processed and presented on the cell surface, tagging the cell as infected for destruction by cytotoxic T cells. So adenoviral vectors are extremely useful if expression of the transgene is required for short periods of time. One promising approach is to deliver large numbers of adenoviral vectors containing genes for enzymes that can activate cell killing, or immunomodulatory genes, to cancer cells. In this case, the cellular immune response against the viral proteins will augment tumour killing. Finally, although immunosuppressive drugs can extend the duration of expression, our goal should be to manipulate the vector and not the patient.

**Adeno-associated viral vectors**

A relative newcomer to the field, adeno-associated virus (AAV) is a simple, non-pathogenic, single-stranded DNA virus. Its two genes (*cap* and *rep*) are sandwiched between inverted terminal repeats that define the beginning and the end of the virus, and contain the packaging sequence[20]. The *cap* gene encodes viral capsid (coat) proteins, and the *rep* gene product is involved in viral replication and integration. AAV needs additional genes to replicate, and these are provided by a helper virus (usually adenovirus or herpes simplex virus).

The virus can infect a variety of cell types, and — in the presence of the *rep* gene product — the viral DNA can integrate preferentially[20] into human chromosome 19. To produce an AAV vector, the *rep* and *cap* genes are replaced with a transgene. Up to $10^{11}$–$10^{12}$ viral particles can be produced per ml, but only one in 100–1,000 particles is infectious. Moreover, preparation of the vector is laborious and, due to the toxic nature of the *rep* gene product and some of the adenoviral helper proteins, we currently have no packaging cells in which all of the proteins can be stably provided. Vector preparations must also be carefully separated from any contaminating adenovirus, and AAV vectors can accommodate only 3.5–4.0 kilobases of foreign DNA — this will exclude larger genes. Finally, we need more information about the immunogenicity of the viral proteins, especially given that 80% of the adult population have circulating antibodies to AAV. These considerations notwithstanding, AAV vectors containing human factor IX complementary DNA have been used to infect liver and muscle cells in immunocompetent mice. The mice produced therapeutic amounts of factor IX protein in their blood for over six months[21,22], confirming the promise of AAV as an *in vivo* gene-therapy vector.

**Other vectors**

Among the other viruses being considered and developed, is herpes simplex virus, which infects cells of the nervous system[23]. The virus contains more than 80 genes, one of which (*IE3*) can be replaced to create the vector. Around $10^8$–$10^9$ viral particles are produced per ml, but the residual proteins are toxic to the target cell. Additional genes can be deleted, allowing more than one transgene to be included. But if essentially all of the viral proteins are deleted (gut-less vectors), only around $10^6$ viral particles are produced per ml. And, again, many people have an immunity to components of herpes simplex virus, having already been infected at some time.

Vaccinia-virus-based vectors have also

been explored, largely for generating vaccines[24]. The Sindbis and Semliki Forest virus is being exploited as a possible cytoplasmic vector[25] which does not integrate into the nucleus. Although most of these systems produce the foreign protein only transiently, they add diversity to the available systems of gene transfer (Table 2).

### Clinical trials

Over half of the 200 clinical trials that have been launched in the United States involve therapeutic approaches to cancer. Nearly 30 of them involve correction of monogenic diseases (Table 1) such as cystic fibrosis, $\alpha_1$-antitrypsin deficiency and severe combined immunodeficiency (SCID). Most of the trials are phase I (safety) studies and, by and large, the existing delivery systems have no major toxicity problems. Moreover, lessons can be learned from previous clinical trials[26,27]. For example, seven years ago two patients were enrolled in a trial to correct deficiencies in adenosine deaminase (ADA, which leads to severe immunodeficiency). One of the patients improved, and makes 25% of normal ADA in her T cells, five years after the last infusion of infected T cells (although she is still treated with PEG–ADA; bovine adenosine deaminase mixed with polyethylene glycol). But in the other patient, the infected T cells could not produce enough of the deficient enzyme.

The efficacy of gene therapy cannot be evaluated until patients are completely taken off alternative treatments (in the above example, PEG–ADA). In another trial[28], weaning a patient away from PEG–ADA reduced the ability of the T cells to respond *in vitro* to a challenge by pathogens. Clearly, in these cases the retroviral vectors were not optimal, and the number of infected blood-progenitor cells was extremely low. However, these trials did help to establish the technology for generating clinical-grade recombinant retroviral particles, the procedures for infection and transplantation, and the protocols for monitoring patients and analysing data. The disappointing outcome probably lies in the inefficient gene-delivery system. We need a system in which the vector — containing the ADA gene — is efficiently delivered to progenitor cells, leading to sustained expression of high levels of the ADA protein. But, encouragingly, despite being repeatedly injected with highly concentrated recombinant viruses, the patients have suffered no untoward effects to date.

### Future prospects

We now need a renewed emphasis on the basic science behind gene therapy — particularly the three intertwined fields of vectors, immunology and cell biology.

All of the available viral vectors arose from understanding the basic biology of the structure and replication of viruses. Clearly, existing vectors need to be streamlined further (see box on page 241), and vectors that target specific types of cell are being developed. The use of antibody fragments, ligands to cell-specific receptors, or chemical modifications to the vector need to be explored systematically. And advances such as the report — published only last week[29] — of the three-dimensional structure of the Env protein from mouse leukaemia virus (a commonly used vector), will facilitate the design of targeted vectors. A better understanding of gene transcription will allow us to design regulatory elements that permit promoter activity to be modulated, and development of tissue-specific enhancer–promoter elements should be vigorously pursued. Finally, we need to begin merging some of the qualities of viral vectors with non-viral vectors, to generate a safe and efficient gene-delivery system.

With respect to immunology, viruses still have many secrets to be unravelled. Viral systems that have evolved to escape immune surveillance can be incorporated into viral vectors. Some of these are being characterized; for example, the adenoviral E3 protein, the herpes simplex ICP47 protein and the cytomegalovirus US11 protein[30]. Systems from other pathogens may also be borrowed and incorporated into vectors. In some cases, the correcting protein will be sensed as foreign, eliciting an immune response. Animal models should help us to understand this and, where necessary, to develop strategies for tolerance.

Cell biology is involved because, in many cases, the goal of gene therapy is to correct differentiated cells, such as epithelial cells in cystic fibrosis and lymphoid cells in ADA deficiency. However, because these cells are continuously replaced there has to be either continued therapy or an attempt to target the stem cells. We first need to develop further the technologies for identifying and isolating these cells, to understand their regulation, and to target infection to them *in vivo*.

So how far have we come since clinical trials began? The promises are still great, and the problems have been identified (and they are surmountable). But what of the prospects? Our view is that, in the not too distant future, gene therapy will become as routine a practice as heart transplants are today. □

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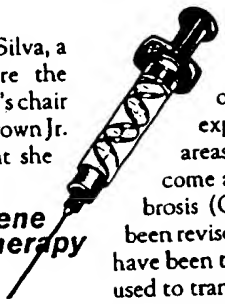
# EXHIBIT E

# Gene Therapy's Growing Pains

With more than 100 clinical trials started and hundreds of millions of dollars at stake, the field is struggling to meet expectations

Last September, when Ashanthi DeSilva, a cheerful 8-year-old, appeared before the House Science Committee, the panel's chair at the time, Representative George Brown Jr. (D–CA), was moved to declare that she was "living proof that a miracle has occurred." DeSilva made history in 1990 when she received the first authorized human gene therapy ever attempted. She had been born with a defective version of the gene that normally makes the essential enzyme adenosine deaminase (ADA)—a condition that, if left untreated, causes a fatal malfunction of the immune system. Four years after receiving her first injection of cells containing functioning ADA genes, Ashanthi, apparently in good health, was chatting with members of Congress.




Since that epic treatment, gene therapy has taken off like a rocket. More than 100 clinical trials, aimed at treating conditions ranging from inherited disorders such as cystic fibrosis to cancer and AIDS, have been given the go-ahead. The National Institutes of Health (NIH) is spending an estimated $200 million a year to develop and test tools and techniques for gene therapy. Private companies have raised hundreds of millions of dollars to enter the field and are now sponsoring most of the clinical trials. Many academic centers have created gene-therapy programs and joined the jockeying for a piece of the action.

Yet in spite of this enthusiasm—bolstered by media hype—all is not well in the world of gene therapy. So far, there has been no unambiguous evidence that genetic treatment has produced therapeutic benefits. Even data from the pioneering ADA trials are not decisive: Ashanthi and the other children who have since been treated with gene therapy are also being given routine injections of synthetic ADA, and these conventional treatments may be responsible for their good health (see box on p. 1051). Gene therapists are still encountering difficulties in transferring genes to adequate numbers of target cells and getting them expressed. This problem afflicts all areas of gene therapy, but it has become acute in efforts to treat cystic fibrosis (CF): Several CF protocols have been revised because of side effects that may have been triggered by the adenovirus agent used to transfer genes, and some researchers say that adenovirus-based therapy for CF must now be rethought (see box on p. 1052).

Faced with such fundamental problems, several biomedical leaders, including NIH Director Harold Varmus, are saying it's time for NIH to pause, examine what gene therapy has accomplished, and determine what role NIH should be playing in the field. "Despite the growing support for gene therapy," Varmus said at a public meeting in May, the field "remains at a very early stage of development. While there are several reports of convincing gene transfer and expression, there is still little or no evidence of therapeutic benefit in patients—or even in animal models." Nor, he added, is there a consensus about which gene delivery systems will be most effective, and he said he wasn't confident the field was choosing the best lines of attack.

Of particular concern to Varmus and some leaders in the field is the possibility that the intense commercial interest in gene therapy is prompting a stampede into clinical trials and pressure for quick results—before the basic science has been worked out. Drew Pardoll, a Johns Hopkins University co-investigator in a gene-therapy trial for prostate cancer, deplores the lack of rigor in many studies. "There's been an emphasis on glitz," he says. "It's produced a culture in which getting into clinical trials—getting into the club—has been more important than getting a meaningful result."


JOHN CRAWFORD/NIH

**High hopes.** The first attempt, in 1990, to correct an ADA gene defect.

These concerns have prompted the most intensive review of this burgeoning field since that first ADA experiment 5 years ago. Earlier this year, Varmus created two high-level panels to advise him on how NIH should proceed. The first, chaired by Inder Verma, a geneticist at the Salk Institute, is looking into NIH's procedures for approving gene-therapy clinical trials (see box on p. 1054). The second, co-chaired by Arno Motulsky, a geneticist at the University of Washington, Seattle, and Stuart Orkin, a hematologist at Harvard University, has been asked to chart a strategy for how NIH should invest in gene therapy, choose areas to emphasize, and help shape guidelines for medical practice. Both panels will issue recommendations by December.

The Motulsky-Orkin panel is drawing a lot of interest—and some nervousness—from gene-therapy researchers in part because Varmus deliberately set it up to take an independent look at the field. Varmus chose its members, he said, for their "stature in the scientific community" and because none is directly involved in running a gene-therapy company or clinical trial.

Varmus's intramural adviser on gene therapy, virologist Nelson Wivel, director of the NIH Office of Recombinant DNA Activities, says he "would not be surprised" if the panel suggests backing off from the heavy emphasis on clinical trials today. Instead, Wivel suggests, the panel may stress the importance of funding basic virology and immunology. "This is the primary question," Wivel says: "Should you be doing [clinical] trials before you've solved other major technical issues," such as making vectors more efficient and less toxic? These recent developments at NIH, the cradle of gene therapy, suggest the soaring enthusiasm for clinical experimentation may be cooling.

### A glass half full?

That enthusiasm is still very visible these days—particularly in the media. "Gene Therapy Techniques Advance as Potential Treatments for Cancer," reported *Genetic Engineering News* on 1 March. "The Birth of a Megamarket," proclaimed *Fortune* on 1 May, featuring Canji Inc., a gene-therapy company in San Diego. "Gene Therapy May One Day Help Doctors Fix Ailing Hearts," announced Johns Hopkins University on 28 July. "Gene Therapy Boosts Radiation Therapy for Cancer," said a University of Chicago press release on 31 July.

Beginning with a wave of media attention

# Jury Still Out on Pioneering Treatment

Every time physician Melissa Elder opens a vial of the enzyme she injects into two young brothers she treats, it costs $2200. Elder says the two boys use a total of five vials a week; it costs more than $40,000 a month to keep them healthy.

These brothers—Rhett, age 4, and Zach, age 2—lack a gene that expresses the enzyme adenosine deaminase (ADA), essential to the immune system. Failure to produce ADA leads to a deadly condition: severe combined immunodeficiency disease. To fend it off and keep infection at bay, Elder, an immunologist at the University of California, San Francisco, treats Rhett and Zach with a synthetic form of the enzyme known as PEG-ADA.* She says the parents are acutely aware of their sons' vulnerability—and of the cost of using PEG-ADA: "The parents lose sleep worrying about what will happen when their insurance reaches its cap." The policy has a limit of $1 million, already half spent.

This is exactly the kind of misfortune gene therapy is meant to prevent. But it hasn't in this case: Zach has received gene therapy to replace missing ADA genes since shortly after he was born. Like the other children who have been given ADA gene therapy in the United States and overseas, he still gets weekly injections of PEG-ADA. Even the two girls who made history 5 years ago as the first patients to receive ADA gene therapy receive PEG-ADA shots. The reason: Physicians have seen other children's immune function decline when PEG-ADA was reduced, and they worry that it would risk the children's health to rely on gene therapy alone.

Elder and other physicians treating the handful of children who have been given gene therapy for ADA deficiency say their patients' health has improved. But as long as the children continue to get PEG-ADA shots, researchers cannot say for sure how much of the credit should go to the gene therapy.

**Proof seeker.** NIH's Blaese aims to prove ADA therapy works.

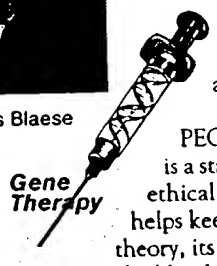
Even principal investigators in the gene therapy trials—Michael Blaese of the National Institutes of Health (NIH) and Donald Kohn of the Los Angeles Children's Hospital—agree that the mixed treatment clouds the role of gene therapy. "There are a lot of questions to be answered," Blaese concedes. But he argues that, in the case of his first two gene-therapy patients, "the experiment was valuable irrespective of whether [the children] were on enzyme or not." He says the experiment proved that it's possible to transfer corrective genes to humans and to get the genes to express ADA "at a very good level" in at least one patient—Ashanthi DeSilva—for several years.

Ashanthi was given her first dose of gene therapy in 1990; a second patient was treated in 1991. Both were also put on PEG-ADA, approved as a standard therapy in 1990 by the Food and Drug Administration. In attempting gene therapy, Blaese and a team at NIH focused at first on T cells circulating in the girls' bloodstream—removing blood, treating T cells with stimulants, inserting a new ADA gene, and infusing the cells back into the patients. Each girl received 11 to 12 treatments. Blood tests conducted 3 years later showed that more than 50% of Ashanthi's circulating T cells contained the new gene, says Blaese.

But, in a telling indication of the hit-or-miss nature of this new technology, only 0.1% to 1% of the other patient's did. Clinical signs have improved in both girls, however. In Ashanthi's case, "it's very hard to say this was due just to enzyme [PEG-ADA]," says Blaese, although he recognizes that in the other case, "there just isn't enough" of the new gene present for it to deserve much credit.

Other researchers say it's easy to overestimate gene therapy's contribution. Ricardo Sorensen, a physician at the Louisiana State University Medical Center who treats ADA-deficient children, notes that the infusion of stimulated T cells alone may have been beneficial for these young patients and that the PEG-ADA must have helped. The best way to sort out what each treatment did, says Sorensen, would be to give T cell therapy, PEG-ADA therapy, and gene therapy independently to patients with similar conditions. Short of that, says Michael Hershfield, the Duke University researcher who developed PEG-ADA, a good way to get an answer would be to withdraw PEG-ADA from children who have received gene therapy and see how they do. That is exactly what Blaese and his colleague Kohn are doing right now.

Together, they have been running an experiment in which Zach and two other ADA-deficient boys were given a new type of ADA gene therapy in 1993, at their birth. In all three cases, researchers removed blood from the children's umbilical cord and attempted to inject an ADA gene into long-lived "stem" cells, which give birth to other blood cells and are relatively abundant in cord blood. The goal: to create a permanent source of ADA-competent T cells. Preliminary data suggest partial success: Up to 10% of their circulating T cells now seem to carry a healthy gene, and Kohn says the hope is that, with time, these healthy cells will accumulate and predominate.




These children have also been receiving PEG-ADA since birth, says Kohn, because "it is a standard therapy, and we felt it wouldn't be ethical to withhold it." However, the PEG-ADA helps keep genetically defective cells alive, and in theory, its use retards the rate at which they can be cleared from the bloodstream to make room for healthy cells. For this reason, Blaese and Kohn are eager to see the boys' PEG-ADA shots curtailed. Since January, Blaese says, the level of PEG-ADA given these three patients has been cut in half (to 30 units per kilogram per week). By the end of the year, he had hoped to cut it close to zero.

But the experiment is not advancing as rapidly as Blaese would like. Physicians for all three boys—including Elder—say they are reluctant to cut the PEG-ADA doses below the present level. Elder, for example, says: "The more PEG-ADA I give, the better the white cell count" and the stronger the immune function. Already the patients' white cell counts have dropped with the initial decline in PEG-ADA doses, although the fraction of "cured" T cells has increased. Physicians are watching closely to see whether the boys can tolerate further reductions before allowing the experiment to proceed. If so, and if the transplanted genes eventually provide all the ADA Zach and the other two boys in this test require, it would be the first unambiguous demonstration that gene therapy has cured a patient's disease.

–E.M.

* Polyethylene glycol–ADA, bovine ADA with artificial surfaces added to prolong life in the bloodstream, manufactured as Adagen by Enzon Inc. of Piscataway, N.J.

generated by NIH's attempt to fix Ashanthi DeSilva's defective ADA gene 5 years ago, encouraging reports like these have swelled to a flood. Most such reports are based on research developments that have yet to be tested in clinical trials, however. And the clinical trials that have been conducted over the past 5 years have yielded very few published results—so few that the Motulsky-Orkin panel will have little hard data to analyze as it tries to figure out how the field is progressing.

NIH's Recombinant DNA Advisory Committee (RAC), which reviews all NIH-funded clinical research protocols for gene therapy, discovered for itself the paucity of data when it established a subcommittee to see where the field is heading. The panel, led by Brian Smith, a Yale University oncologist and RAC member, and NIH staffer Debra Wilson, scanned all trials approved by the RAC and the Food and Drug Administration (FDA) through June 1995. The panel found little concrete information on the results of these trials, but it did paint a remarkable picture of how rapidly the field has grown—both in terms of the numbers of trials and the wide range of disorders gene therapists are boldly trying to treat.

The RAC team found that 567 patients are involved in 106 RAC-approved experiments. Almost half (268) are new recruits, having entered trials since December 1994. Only a small fraction of these experiments are aimed at correcting defective genes. Instead, most protocols aim to induce specific cells, such as cancer cells or cells infected by HIV, to produce proteins that would make them vulnerable to attack by the immune system. Others are attempting to use gene therapy as an adjunct to traditional chemotherapy for cancer (see chart on next page).

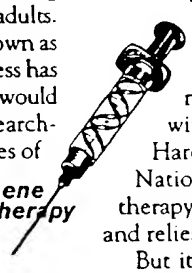
The field, in short, has moved a long way from the popular notion of gene therapy as a cure for genetic disease. Indeed, the RAC panel identified only 20 trials focusing on single-gene deficiencies such as ADA. Of these, 11 aim to replace the defective chloride transport gene that causes cystic fibrosis, using an adenovirus vector to shuttle functioning genes into a patient's lung cells. Three other trials aim to treat Gaucher disease, a metabolic disorder; single trials are aimed at each of six other rare diseases including ADA deficiency. Little has been published from these efforts: Only preliminary data have seen the light of day in peer-reviewed journals.

In contrast to the few efforts aimed at single-gene disorders, almost half the 106 trials are aimed at cancer. One reason for the

## The Trouble With Vectors

Cystic fibrosis (CF) is a lethal inherited disease for which gene therapy offers a rare hope of relief. CF patients—of whom there are more than 30,000 in the United States—lack a gene that enables cells to process the chloride ion, causing their lungs to be plagued by mucus and infection. Gene therapy's promise is that one day it may be possible to replace defective genes with healthy ones, lengthening the lives of CF patients, who generally die as children or young adults.

Already, researchers have transferred a working gene (known as *CFTR*) into the surface airway cells of lab animals. This success has inspired 11 human trials. But any expectation that these tests would quickly demonstrate therapeutic benefits has dwindled as researchers have run into problems in transferring sufficient quantities of the *CFTR* gene into patients' cells. In addition, the virus vector they are using as the transfer agent has provoked an immune reaction in some patients.




CF researchers are not alone in encountering such difficulties. Indeed, right from the start, gene therapists have recognized that their central challenge would be to find safe vectors capable of transporting genes efficiently into target cells—and getting the cells to express the genes once they are inserted. Although there have been promising developments in some areas, it remains the central challenge for every area of gene therapy. Francis Collins, director of the National Center for Human Genome Research (NCHGR), sums up the situation bluntly: "None of the currently available techniques is clinically useful for systemic gene delivery," the kind that can provide a permanent cure. For that reason, NCHGR has joined in a major program to improve vectors and make them available to clinicians (*Science*, 11 August, p. 751).

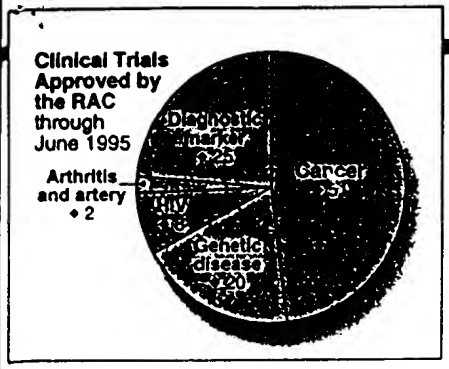
The most popular vector used so far is one based on a retrovirus that normally infects mice. A crippled version of this retrovirus, loaded with therapeutic genes, has been used in 76 of the 106 human trials approved to date, most of which involve patients with cancer or HIV. This mouse virus is the most efficient agent yet identified for transferring genes, although rates of transfer and expression vary dramatically in different patients (see p. 1051).

The stark variation among patients isn't the only problem. Another is that retroviruses insert genes only into cells that are actively dividing and growing, such as T cells. This rules out their use for treating diseases such as CF, where the target cells aren't dividing. A second drawback is that retroviruses insert themselves randomly into host DNA, posing what's thought to be a small—but real—risk of cancer. If a retrovirus gene should settle alongside an oncogene or tumor suppressor gene, it might trigger tumor formation by turning on or off the native gene. For these reasons, retroviral vectors have been used in "ex vivo" procedures—in which cells are removed from the patient, treated, and replaced—and when increased risk of cancer is not considered an obstacle to therapy.

In contrast to those trials, therapy for CF patients has relied primarily on a vector based on a crippled adenovirus. This DNA virus infects 75% of young people, usually without causing illness, according to adenovirus expert Harold Ginsberg, emeritus of Columbia University, now at the National Institutes of Health (NIH). It's attractive for CF therapy because it seeks the lungs. It penetrates nondividing cells and relies on these host cells to express viral DNA.

But it, too, has drawbacks. Adenovirus genes express proteins that trigger immune responses. In consequence, large concentrations of wild virus—and even crippled virus—provoke inflammation along with an immune attack that neutralizes cells containing adenovirus genes. For this reason, the effects of adenovirus vector therapy are likely to be short-lived, lasting about 6 weeks. And because the immune system "remembers" antigens and attacks them with extra vigor on a second encounter, repeat dosing with adenovirus vector seems impractical at present—unless a strong immune response is desired, as in some types of cancer therapy.

Research on CF gene therapy by Richard Boucher and colleagues at the University of North Carolina, Chapel Hill, indicates that clinicians using adenovirus as a vector are caught between two problems. When administered at low concentrations it is inefficient: The virus doesn't get into many human nasal cavity or airway cells, and few cells express the corrected *CFTR* gene. At high doses, however, it appears to cause acute inflammation, Boucher says. He notes that three or four CF gene therapy trials have been compelled to stop or adjust doses to deal with acute reactions in patients. Some researchers, such Ronald Crystal, who pioneered this field at NIH and is now at the Cornell University Medical Center in New York, think past problems with CF therapy may not involve fundamental issues so much as a need to find the right way to deliver existing materials. But Boucher and Ginsberg believe immunogenicity has been and continues to be a fundamental problem.




**Broad focus.** The majority of trials now target diseases with large patient populations.

growing emphasis on this disease, says Wivel, is that private investment in gene therapy is increasing, and companies can't justify large R&D expenses unless they can expect to treat large patient populations. Another reason is that these patients often have no alternatives in conventional medicine and are therefore eligible for experimental therapy.

Thirty of the 51 cancer trials are designed to insert into tumor cells a gene expressing a substance such as the lymphokine interleukin-2 (an immune-system signaling molecule), in the expectation that it will stimulate a natural immune attack on the tumor cells. Another 11 studies aim to induce tumor cells to express the herpesvirus protein thymidine kinase, which makes them vulnerable to treatment with the drug gancyclovir. The remaining 10 trials test three other strategies, including four trials that seek to stop cancer by activating tumor suppressor genes. No results have yet been published from these trials.

Another fast-growing area is gene therapy for AIDS. Indeed, the majority of patients enrolled in clinical trials approved in the first half of 1995—168 out of a total of 268—are participating in tests of an anti-HIV therapy sponsored by Viagene Inc. of San Diego. (These include a trial approved only by FDA; private trials need not obtain RAC approval.)

Viagene has focused on a succession of strategies in at least four RAC-approved HIV trials. These studies aim to put genes that express HIV proteins into some of a patient's cells, in the hope that the cells will express antigens that will prime the immune system to attack infected cells carrying the same antigens. In addition to Viagene's trials, five others go after HIV with other strategies: They seek to disrupt viral functions by creating decoy molecules to compete with, sequester, or cleave products produced by HIV, or they try to cause HIV-infected cells to express thymidine kinase or other molecules that make them targetable by chemical attack. Clinical results have not yet been published from any of these trials.

These two vector types—retrovirus and adenovirus—account for more than 85% of those used in clinical trials. But leading researchers and a few companies are looking for other vehicles. For example, Joseph Glorioso, director of the gene-therapy program at the University of Pittsburgh, is focusing on herpesvirus. It infects the central nervous system and carries a remarkable "latency gene" that hides it from immune attack. In theory, herpesvirus vector could be used to insert DNA into the nervous system. But it is difficult to manipulate and may have hidden risks. Eventually, Glorioso hopes to seek approval to use it for cancer therapy.

R. Jude Samulski, leader of the gene-therapy program at the University of North Carolina, is investigating adeno-associated virus (AAV). It has no known toxicities and replicates only in the presence of a "helper virus," making it look very safe. It may have other advantages, says Samulski: It is simple, and its "life cycle suggests it may be able to persist and deliver genes for a long time."

But Samulski acknowledges a common criticism: AAV is difficult to produce in large quantities. One leading gene-therapy researcher who asked not to be named claims that current AAV technology is inefficient and expensive, adding: "AAV is like an onion—the more layers you peel off, the more you cry." So far, only one human trial using AAV—for treatment of cystic fibrosis—has been approved; no data are yet available. But Samulski predicts, "You'll see more and more AAV protocols."

Viagene Inc. of San Diego is investigating sindbis—an African virus that infects the nervous system—as a new vector. Its virtues include a unique and highly productive method of replication. Other groups are looking into HIV as a vector, although probably only for treating HIV patients.

The hottest alternatives to viruses are oily substances known as cationic liposomes. These concoctions, which come in many varieties, can slip DNA into the cell's nucleus and cause genes to be expressed. One skilled user of the technology, Gary Nabel, a Howard Hughes Medical Institute investigator at the University of Michigan, predicts liposomes will improve dramatically in the next few years, increasing levels of gene expression by "an order of magnitude or more," and that they will quickly be adapted for clinical use. In addition to liposomes, two other nonviral vectors have been adopted for clinical trials: direct injection of plasmid DNA into the muscle (two trials) and air-gun injection of a DNA-coated pellet (one trial).

These examples should make clear that there is as yet no perfect vector. And many researchers say we shouldn't expect one: Instead, there will be a confusing array of viral bits and pieces, combined with other gene-transfer agents, all of them used in custom tools designed for specific applications. But if a perfect vector were to be created, it might look something like the one being pursued by the intramural research staff at NCHGR.

NCHGR has launched a project, led by Melissa Rosenfeld and Paul Liu, to develop what they call a "human artificial chromosome." The idea is to create a synthetic 25th chromosome, big enough to transport whole "suites" of genes into the nucleus of a target cell, including all the regulatory sequences that surround a critical gene. Collins told a review panel in May that NCHGR is collaborating with a few extramural groups in an "intense" effort to push this "high-risk" project forward. But a staffer notes that it hasn't yet achieved "proof of principle." For the present, Collins observed, "the paucity of clinically acceptable gene transfer techniques severely limits the potential applications of gene therapy."

–E.M.

**VECTORS IN RAC-APPROVED CLINICAL TRIALS**

SOURCE: RAC

| Vector | Number of clinical trials | Pluses | Minuses |
|---|---|---|---|
| **VIRAL** | | | |
| Retrovirus | 76 | Efficient transfer<br>Easy to make | Small capacity<br>Random DNA insertion<br>Dividing cells only<br>Replication risk |
| Adenovirus | 15 | Nondividing cells<br>Possibly targetable | Immunogenic<br>Replication risk |
| Adeno-associated virus | 1 | Nonimmunogenic | Small capacity<br>Hard to make |
| Herpesvirus | 0 | Nonimmunogenic<br>Targets CNS | Risks unclear<br>Hard to make |
| **NONVIRAL** | | | |
| Liposomes | 12 | No replication<br>Nonimmunogenic | Low efficiency |
| Naked particle-mediated DNA | 3 | No replication risk<br>Nonimmunogenic | Low targetability<br>Low efficiency |

## RAC's Identity Crisis

From the start, gene therapy has been one of the most contentious fields in biomedicine. In the early days, debate focused on safety—on the possibility, for example, that engineered DNA might create novel infectious viruses or trigger new forms of cancer. To minimize such risks and reassure the public, the National Institutes of Health (NIH) beginning in 1980 asked all government-funded gene therapy researchers to submit protocols for prior approval by a public panel.

Today, these reviews are carried out by NIH's Recombinant DNA Advisory Committee (RAC), a mixed group of 20 scientists and nonscientists who meet quarterly at NIH. RAC has voted on virtually every gene therapy trial in the United States.

Now, after 5 years of clinical experimentation and no evidence that gene therapy poses a general risk to the public, fears are fading and, with them, the justification for RAC. Some leaders in gene therapy—especially researchers eager to get experiments—launched and companies with large sums hanging on clinical trials—are saying it's time for RAC to think about retiring. They point out that the law already requires the Food and Drug Administration (FDA) to monitor clinical trials and clear therapeutic products for safety and efficacy, which means that gene therapy has to pass two federal checkpoints.

Stephen Marcus, an executive at Genetic Therapy, Inc. of Gaithersburg, Maryland, says, for example, that RAC delays by 2 months or more decisions ultimately made at the FDA. "It may be a little hyperbolic" to suggest that lives are being lost as a result, Marcus says, "but the concept is there." Likewise, Thomas Reynolds of Targeted Genetics in Seattle complains that RAC has become a "bottleneck." He'd rather see it focus on novel issues, like germ-line therapy, new vector systems, or new disease targets"—not on reviews of individual protocols.

Members of the RAC themselves have also expressed uncertainty about the role they're expected to play, NIH Director Harold Varmus has observed. While public members of the panel tend to focus on safety and ethics, those with expertise in biology often zero in on technical aspects of proposals that they find inadequate. RAC has thus suffered from a split personality.

To clarify the committee's proper role, Varmus has taken a couple of steps in the past year. First, he has asked RAC's staff and the FDA to work out a unified review process, now being put into effect, that may allow researchers to submit a single application for review by both agencies. Only those that raise new technical or ethical issues would be debated by the RAC.

Second, Varmus has appointed a special ad hoc study group—headed by oncogene researcher Inder Verma of the Salk Institute in La Jolla, California—to consider how NIH should review gene therapy in the future. One of the big questions to be addressed is: Who, if anyone, should scrutinize clinical trials for scientific value? Officially, that isn't RAC's job, although expert members find it hard not to comment on technical quality. The Verma panel will deliver its recommendations on this and other broad questions about how NIH should handle gene therapy trials to Varmus by December.

—E.M.

Most of the other trials reviewed by RAC are not aimed at delivering therapy: They are designed to tag specific cells with genetic markers to provide information about the fate of the cells. When RAC members sifted through the catalog of these "gene marking" trials in June, they found that although this area gets little public attention, it is in fact scientifically the most encouraging area. Smith says they have produced at least four peer-reviewed publications laden with "hard data." The research has shown, for example, that cancer relapse following autologous bone marrow transplants—in which a patient's bone marrow is removed before intensive chemotherapy and later replaced—often is caused by tumor cells that survive in the marrow. It indicates that clinical research should zero in on ways to purge tumor cells from the marrow.

Whether this overall picture is judged positive depends in large measure on who's being asked. Pioneer gene therapists and industry leaders tend to view the explosion of trials as evidence of progress. Independent academics, on the other hand, often see the glass as half empty. But both sides can agree that, at the least, the field isn't harming its patients. Clinical trials, says Smith, have shown few signs of toxicity and no hints of runaway genetic mutations: "There are no three-headed cows" of the kind anticipated in "*National Enquirer*–land," he jokes. But the disappointing news, Smith finds, is that so far only hints of therapeutic benefit have appeared.

Wivel notes that nearly all the gene-therapy trials so far have been "phase I" trials, designed to test safety rather than efficacy. So they can't really be judged on effectiveness. But that hasn't discouraged some gene-therapy leaders from trying. Stephen Marcus, a vice president at Genetic Therapy Inc. of Gaithersburg, Maryland, cites a brain cancer patient who, after surgery for glioblastoma, was treated with GTI's anticancer gene therapy and has survived for more than 2 years. This is almost unheard of, Marcus says, and is clearly "a case where there is some evidence of effectiveness." He notes, however, that "we realize this is anecdotal."

VICKI VALERIO/THE PHILADELPHIA INQUIRER

**Back to basics.** James Wilson wants less hype, more research.

But the RAC members who reviewed cancer trials—Robert Erickson of the University of Arizona and R. Jude Samulski of the University of North Carolina—deemed it "too early" to reach any conclusions. Erickson found several unpublished reports that gene therapy had reduced tumors—but noted that other, simpler therapies had produced similar reports in the past. Samulski pointed to a common theme running through the cancer studies that raised some concern: low rates of gene transfer.

Indeed, difficulties in getting genes transferred efficiently to target cells—and getting them expressed—remain a nagging problem for the entire field. Virus-based vectors have been the most efficient for inserting genes into cells in the lab, but they have run into problems in the clinic. Often the fraction of cells receiving the new gene is low, particularly if these targets of gene therapy are long-lived "stem" cells that give birth to other cells. Researchers say it has been difficult to achieve a 1% rate of gene transfer into stem cells, for reasons not fully understood. And even when genes are inserted in stem cells, they may not be active in second-generation cells, yielding less-than-adequate therapy.

Boosting the rate of gene transfer by increasing the concentration of vector or dosing patients repeatedly may create another problem, however: It may stimulate the immune system to attack and neutralize the therapy-bearing cells. Francis Collins, director of NIH's National Center for

man Genome Research, told the Motulsky-Orkin panel in May that "many problems must be solved before gene therapy will be useful for more than the rare application."

### Voting with their checkbooks

Academic researchers are still grappling with many fundamental issues in gene therapy. But industry leaders and their financial agents are gung-ho. Investors have poured hundreds of millions of dollars over the past 5 years into gene-therapy companies, drawn by hopes of blockbuster discoveries. And big companies are now getting into the act. Late last year, Switzerland's Ciba-Geigy Ltd. acquired a 49.5% share of Chiron Corp. of Emeryville, California, which then turned around in April 1995 and began buying Viagene. Less than 3 months later, another Swiss pharmaceutical giant, Sandoz AG, bought GTI—an investment that gives Sandoz rights to GTI's broad patent for "ex vivo" therapy, in which cells are removed from the patient, given new genes, and replaced (*Science*, 31 March, p. 1899). Also last fall, the French company Rhône-Poulenc Rorer struck agreements with a network of small companies to gain access to the latest research (*Science*, 18 November 1994, p. 1151).

One result of this burgeoning investment is that private companies have come to dominate clinical trials of experimental gene therapies. By June, according to the RAC, 13 firms had won approval to run at least 34 gene-therapy trials—so that now, 60% of all therapeutic trials are privately funded. Industry also plays an indirect role in physician-sponsored trials, supplying vectors at little or no cost.

This trend is worrying some leaders in the field, who say biotech companies are forcing the pace and direction of research, and not always in ways anchored in the best science. Varmus, for example, says that while it's "a good thing" that investors are willing to pick up the tab for "very expensive" clinical experiments, these trials absorb "a lot of resources and talent," and he isn't sure that they "are scientifically as worthy as other things that could be done." He's concerned about understanding the biology of viruses used to transfer genes and of the immune reactions they provoke.

Varmus isn't alone in expressing concerns. James Wilson, director of the Institute for Gene Therapy at the University of Pennsylvania, says private funding is important, but he worries that expectations may be raised prematurely. People who invest in gene therapy anticipate a big payoff, but they may not realize how long it will take, Wilson says. "The actual vectors—how we're going to practice our trade—haven't been discovered" yet, he notes, "so it may be early for the impatience of venture capital–supported biotech."

This commercial pressure may also account for some of the hype surrounding developments in gene therapy, says Wilson. If you're the leader of a gene-therapy company, "you try to put as positive a spin as you possibly can" on every step of the research process, he notes, "because you have to create promise out of what you have—that's your value." But, Wilson says, "that's not what we need right now." What the field needs is "a lot of basic research on vectors and cell biology."

Pardoll of Hopkins is equally critical; he says that in the rush to get trials approved, "biological principles are not well thought out—especially immunological principles." Varmus says this happens because the main concern of small companies is to survive, and "one way to survive is to have a clinical trial—show that you're actually on the scoreboard." But promoting the company doesn't necessarily promote gene therapy, Varmus notes: "We're not talking about an industry that's in an advanced state of competence."

Another effect of commercial investment, some researchers say, has been to channel energy into intellectual property disputes and turf battles. For example, Dusty Miller, a virologist at the Fred Hutchinson Cancer Research Center in Seattle, argues that the gene-therapy patent issued to NIH and GTI in April will have a "chilling effect" on research. The patent covers all forms of ex vivo therapy. Miller—who was among those involved in the research that led to this patent but was not named as a co-inventor—calls it "the patent from hell" because it's so broad. He thinks it may discourage newcomers and stifle collaboration. When Miller made this comment at a RAC meeting in June, GTI President James Barrett rose to say the company considers the patent "valid" and will negotiate reasonable terms that are "idiosyncratic" for each use.

Academic scientists may think it's too early to be talking about financial returns, but not company executives and some industry analysts. Take Wall Street biotech analyst Jeffrey Swarz of the investment bank CS First Boston. Swarz delivered an enthusiastic assessment of the field at last year's congressional hearings and was equally bullish in a recent interview with *Science*. Gene therapy for cystic fibrosis, he said, "has been successful; ADA disease has been successful; brain cancer has been successful. ... So far, the technology looks fabulous." He predicts a gene-therapy product will reach the market by next year.

At a recent meeting in Washington, D.C., organized by the Institute for International Research, gene-therapy business chiefs were asked when they thought their industry's first product would hit the market. Few were as optimistic as Swarz, but the forecasts ranged from very soon—in 1997, according to David Nance, president of Introgen Therapeutics of Austin, Texas—to reasonably soon—in 2000, according to Harvey Berger, chair of Ariad Pharmaceuticals in Cambridge, Massachusetts. One attendee, Mark Edwards, managing director of Recombinant Capital, an independent San Francisco firm that analyzes biotech companies, was less ebullient, saying he didn't expect a commercial product until 2003. Whether one's an optimist or not, concluded Berger, "we've got to make sure the biology matches the enthusiasm."

Many academic gene therapists agree with Berger, and some have said they hope the critical review Varmus has ordered from the Motulsky-Orkin panel will cut through the hype that surrounds the field and inform the public that it could be many years before the money invested in clinical trials yields a product. "It may be time for some realism," says Michael Knowles of the University of North Carolina's cystic fibrosis program. Adds Joe Glorioso, director of the University of Pittsburgh's gene-therapy program: "We just can't be wimpy about this; we have to be in for the long haul."

–Eliot Marshall

**U.S. GENE THERAPY TRIALS SPONSORED BY INDUSTRY**

| Company Sponsor | Founded | Capital ($millions) | No. of Trials | Topic |
|---|---|---|---|---|
| Applied Immune Sciences | 1982 | 209 | 1 | Cancer |
| Canji | 1990 | 21 | 1 | Cancer |
| Cell Genesys | 1988 | 103 | 1 | HIV |
| GenVec | 1992 | 20 | 1 | Cystic fibrosis |
| GeneMedicine | 1992 | 50 | 1 | Alpha-1 anti-trypsin |
| Genetic Therapy | 1986 | 103 | 6 | Cancer, CF, Gaucher |
| Genzyme | 1981 | 74 | 5 | Cancer, CF, Gaucher |
| Immune Response | 1986 | 128 | 1 | Cancer |
| Ingenex | 1992 | 5 | 25 | Cancer |
| Introgen Therapeutics | 1993 | NA | 3 | Cancer |
| Somatix Therapy | 1979 | 102 | 3 | Cancer |
| Targeted Genetics | 1989 | 46 | 1 | HIV |
| Viagene | 1987 | 106 | 6 | Cancer, HIV |
| Vical | 1987 | 46 | 4 | Cancer, HIV |

SOURCE: COMPANY DATA AND RAC

# EXHIBIT F

# REPORT AND RECOMMENDATIONS OF THE PANEL TO ASSESS THE NIH INVESTMENT IN RESEARCH ON GENE THERAPY

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## Executive Summary of Findings and Recommendations

Dr. Harold Varmus, Director, National Institutes of Health (NIH), appointed an *ad hoc* committee to assess the current status and promise of gene therapy and provide recommendations regarding future NIH-sponsored research in this area. The Panel was asked specifically to comment on how funds and efforts should be distributed among various research areas and what funding mechanisms would be most effective in meeting research goals.

**The Panel finds that**:

1. Somatic gene therapy is a logical and natural progression in the application of fundamental biomedical science to medicine and offers extraordinary potential, in the long-term, for the management and correction of human disease, including inherited and acquired disorders, cancer, and AIDS. The concept that gene transfer might be used to treat disease is founded on the remarkable advances of the past two decades in recombinant DNA technology. The types of diseases under consideration for gene therapy are diverse; hence, many different treatment strategies are being investigated, each with its own set of scientific and clinical challenges.

2. While the expectations and the promise of gene therapy are great, clinical efficacy has not been definitively demonstrated at this time in any gene therapy protocol, despite anecdotal claims of successful therapy and the initiation of more than 100 Recombinant DNA Advisory Committee (RAC)-approved protocols.

3. Significant problems remain in all basic aspects of gene therapy. Major difficulties at the basic level include shortcomings in all current gene transfer vectors and an inadequate understanding of the biological interaction of these vectors with the host.

4. In the enthusiasm to proceed to clinical trials, basic studies of disease pathophysiology, which are likely to be critical to the eventual success of gene therapy, have not been given adequate attention. Such studies can lead to better definition of the important target cell(s) and to more effective design of the therapeutic approach. They often can be carried out in appropriate animal models. Pathophysiologic studies may also suggest alternative treatment strategies.

5. There is a clear and legitimate need for clinical studies to evaluate various aspects of gene therapy

approaches. Although animal investigations are often valuable, it is not always possible to extrapolate directly from animal experiments to human studies. Indeed, in some cases, such as cystic fibrosis, cancer, and AIDS, animal models do not satisfactorily mimic the major manifestations of the corresponding human disease. Clinical studies represent not only practical implementation of basic discoveries, but also critical experiments which refine and define new questions to be addressed by non-clinical investigation.

6. Interpretation of the results of many gene therapy protocols has been hindered by a very low frequency of gene transfer, reliance on qualitative rather than quantitative assessments of gene transfer and expression, lack of suitable controls, and lack of rigorously defined biochemical or disease endpoints. The impression of the Panel is that only a minority of clinical studies, illustrated by some gene marking experiments, have been designed to yield useful basic information.

7. Overselling of the results of laboratory and clinical studies by investigators and their sponsors--be they academic, federal, or industrial--has led to the mistaken and widespread perception that gene therapy is further developed and more successful than it actually is. Such inaccurate portrayals threaten confidence in the integrity of the field and may ultimately hinder progress toward successful application of gene therapy to human disease.

**Based on these findings, the Panel recommends the following:**

1. In order to confront the major outstanding obstacles to successful somatic gene therapy, greater focus on basic aspects of gene transfer, and gene expression within the context of gene transfer approaches, is required. Such efforts need to be applied to improving vectors for gene delivery, enhancing and maintaining high level expression of genes transferred to somatic cells, achieving tissue-specific and regulated expression of transferred genes, and directing gene transfer to specific cell types. To stimulate innovative research, the Panel recommends the use of interdisciplinary workshops, specific program announcements in these areas, and the use of short-term, pilot grants for testing new ideas and for encouraging investigators from other areas to enter the field of gene therapy.

2. To address important biological questions and provide a basis for the discovery of alternative treatment modalities, the Panel recommends increased emphasis on research dealing with the mechanisms of disease pathogenesis, further development of animal models of disease, enhanced use of preclinical gene therapy approaches in these models, and greater study of stem cell biology in diverse organ systems.

3. Strict adherence to high standards for excellence in clinical protocols must be demanded of investigators. Gene therapy protocols need to meet the same high standards required for all forms of translational (or clinical) research, whatever the enthusiasm for this (or any other) treatment approach.

4. To enhance the overall level of research in this area, the Panel recommends that NIH support broad interdisciplinary postdoctoral training of M.D. and Ph.D. investigators at the interface of clinical and basic science. Mechanisms for physician training in this area might include use of career development awards based on a program announcement in gene therapy.

5. Investigators in the field and their supporters need to be more restrained in their public discussion of findings, publications, and immediate prospects for the successful implementation of gene therapy approaches. The Panel recommends a concerted effort on the part of scientists, clinicians, science

writers, research advocates, research institutions, industry, and the press to inform the public about not only the extraordinary promise of gene therapy, but also its current limitations.

6. NIH has already provided an appropriate initial investment in gene therapy. Future gene therapy research should compete with other forms of biomedical research for funding under stringent peer review. Only with fair, yet critical, peer review will high standards be met and maintained. The Panel specifically does not recommend special gene therapy study sections, expansion of existing center programs in gene therapy, or expansion of the recently funded core vector production program. To ensure that the level of support remains appropriate, the NIH investment in this field should be reexamined periodically.

7. To enhance the contribution of industry to the field, the Panel recommends that NIH encourage collaborative arrangements between academic institutions and industry that complement NIH-supported research, and also implement mechanisms that facilitate the distribution and testing of vectors and adjunct materials for use in clinical studies.

8. In an effort to improve gene therapy research and reduce duplication of effort, the Panel urges better coordination and scientific review of such research throughout the NIH Intramural Program. In addition, NIH Institute Directors should resist pressures to include gene therapy research in their portfolios (either Intramural or Extramural) to "round out" their programs or compete with other Institutes. Instead, they should include such research only when there are compelling scientific reasons to go forward. Institute Directors should take the lead, where it seems appropriate, to focus efforts on improvement of diagnosis and understanding of disease pathogenesis and await further developments in vector technology before expanding clinical gene therapy programs.

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## Introduction

Gene therapy is a set of approaches to the treatment of human disease based on the transfer of genetic material (DNA) into an individual. Gene delivery can be achieved either by direct administration of genecontaining viruses or DNA to blood or tissues, or indirectly through the introduction of cells manipulated in the laboratory to harbor foreign DNA. As a sophisticated extension of conventional medical therapy, gene therapy attempts to treat disease in an individual patient by the administration of DNA rather than a drug. Because only somatic cells, and not germ cells (eggs and sperm), are the target of these efforts, gene transfer affects only the individuals under treatment and not their offspring. Therapy directed to germ cells, which would represent a radical departure in the approach to managing disease, is not considered further in this report.

Since genetic material is the putative therapeutic agent, some observers view gene therapy as qualitatively different from other forms of treatment. Seen from a broader perspective, however, somatic gene therapy reflects a natural progression in the application of biomedical science to medicine. In altering the genetic material of somatic cells, gene therapy may correct the underlying specific disease pathophysiology. In some instances, it may offer the potential of a onetime cure for devastating, inherited disorders. In principle, gene therapy should be applicable to many diseases for which current therapeutic approaches are ineffective or where the prospects for effective treatment appear exceedingly low.

Five years have elapsed since the first patients received gene modified cells at the NIH. Since then, the field of gene therapy has attracted increased attention in scientific, medical, media, and lay circles. As of June 1995, 106 clinical protocols involving gene transfer were approved by the NIH Recombinant Advisory Committee (RAC). Indeed, a total of 597 subjects have already undergone gene transfer experiments. Currently, NIH provides approximately $200,000,000 per year for research related to gene therapy. Industrial support of gene therapy research has grown steadily, such that it now is estimated to exceed that of the NIH and underwrites a major proportion of approved clinical protocols. With this high level of current activity the young field of gene therapy is the focus of attention and scrutiny as a frontier of modern medicine.

To advise Dr. Harold Varmus, Director of the NIH, the Panel to Assess the NIH Investment in Research on Gene Therapy (see Appendix A) heard presentations from NIH Institute Directors, basic researchers, and clinical investigators from academic and federal institutions and from the private sector (see Appendix B). The Panel also reviewed recent basic and clinical research in gene therapy.

Panel members are unanimous in recognizing the extraordinary potential, in the longterm, of gene therapy for managing and correcting human disease. Integrating efficacious and workable gene therapy procedures into the health care system would signal a major development in medicine, comparable to past milestones, such as the introduction of aseptic techniques, antibiotics, vaccines, and tissue transplantation. The realization of this long-term goal requires proper development of its scientific underpinnings and validation of its utility to patients with carefully designed, controlled, and evaluated clinical trials.

Although expectations have been great-fueled by the escalating enthusiasm of some investigators, industrial sponsors, and members of the media-it must be recognized that clinical efficacy in human patients has yet to be clearly established for any gene therapy protocol. This sobering reality highlights the challenge of bringing this, or any other, complex technology to clinical practice. Typically, many years are required before new therapies are proved successful. For example, transplantation of bone marrow and other organs--now an accepted therapy for lifethreatening diseases-required more than two decades of development during which frequent failures often provoked widespread skepticism. At this early stage in the development of gene therapy, the Panel considered the following issues:

- Is the science underlying the application of gene therapy sufficiently mature to justify rapid and widespread clinical testing? What areas of research need particular development?
- Have the clinical trials to date been appropriately designed to be maximally informative? Should stricter standards be adopted?
- Is there an appropriate balance between basic and clinical research supported by the NIH?
- Are training, research, and resource support mechanisms optimal for nurturing this young field? Should special, targeted research and/or training grant mechanisms be instituted?
- What relationships among academic, federal (i.e., NIH), and industrial institutions would best facilitate the development of the clinical applications of gene therapy?
- What is the impact of the manner in which investigators in the field and their supporters portray their activities to the scientific community and the public?

In its review the Panel has identified significant problems that need to be addressed. Its recommendations are based on the view that shortcomings must be frankly acknowledged and overcome to realize the full promise of gene therapy.

## The rationale for gene therapy of human disease

The concept that gene transfer might be applied to treat disease is founded on the extraordinary advances of the past two decades in the area of recombinant DNA technology. Current methods permit rapid identification and facile manipulation of genes, better enabling investigators to determine the molecular basis of disease and to examine cellular physiology from a molecular perspective. The potential use of gene transfer to treat disease, therefore, is a natural extension of recent fundamental biomedical research.

The types of diseases under consideration for somatic gene therapy are diverse, and have many different underlying causes. Accordingly, the rationales and strategies for treating particular diseases are varied. To assess gene therapy's prospects and status, we, therefore, distinguish among major disease categories.

- **Single-gene inherited disorders:** Many inherited disorders result from mutation of a single gene (hence, singlegene [monogenic] disorders). While individually infrequent in the population, this category as a whole contributes significantly to the chronic disease burden, and includes sickle cell anemia, hemophilias, inherited immune deficiency disorders such as adenosine deaminase deficiency, hypercholesterolemia due to defects in the LDLreceptor, and cystic fibrosis. In many instances singlegene disorders are a direct consequence of loss of function of the relevant protein, such that its replacement (or mere addition to the cell) would be curative. This is the most straightforward application of somatic gene therapy and may be entertained once the mutant gene has been identified and its normal counterpart isolated. Delivery of a normal factor VIII gene to a patient with hemophilia is an example. In some instances, the mutant protein participates more indirectly in cellular pathology, such as in sickle cell anemia where a variant globin causes hemoglobin to polymerize under low oxygen tension, thereby damaging the red blood cell. In this situation, gene transfer and expression of a normal globin chain is still expected to benefit the patient. In yet other instances, such as in dominantly inherited connective tissue disorders in which the presence of an abnormal molecule interferes with normal tissue development and function, only selective silencing of the mutant gene would be expected to be of benefit to the patient.

  Although "gene addition" is the simplest strategy for somatic gene therapy, several practical difficulties need to be addressed. Particularly important among these is the need in many instances to deliver the appropriate gene to a specific cell type or tissue. Other challenges includes gaining access to the relevant cell type for correction, assessing the total fraction of cells in a tissue that need to be corrected, achieving the level of expression required for correction, and regulating expression of the added gene once it is transferred into appropriate target cells.

- **More common, multifactorial disorders:** For a variety of more common diseases (e.g., coronary heart disease, diabetes), typically several genes are involved, making a single gene mechanism exceptional. Knowledge of pathophysiology is beginning to suggest how in particular instances the introduction of specific genes might reverse or retard disease processes at the cellular level. This general approach may prove effective regardless of genetic etiology and without the need to replace a single, missing gene product. For instance, in restenosis following angioplasty, local

transfer into vascular cells of genes reducing proliferative and thrombotic processes might prevent reocclusion.

The possibilities for gene transfer as a treatment for common multifactorial diseases are vast. The precise approach needs to be assessed in each instance by considering how specific gene products influence cellular physiology. We can expect many different, sometimes speculative, strategies to be proposed. Each will need to be judged in comparison with conventional treatment approaches.

- **Cancer:** Studies of the past two decades have established cancer as a genetic disease at the cellular level. Cancers arise through a multistage process driven by inherited and relatively frequent somatic mutation of cellular genes, followed by clonal selection of variant cells with increasingly aggressive growth properties. At least three important classes of genes-protooncogenes, tumor suppressor genes, and DNA repair genes-are targeted by mutations. In less than five percent of all individuals with cancer, and a greater percentage of those developing cancer at a younger age, germline mutation of a tumor suppressor or DNA repair gene is a primary determinant for cancer development. However, in contrast to the gene therapy approaches being considered for typical inherited disorders in which a gene product is missing, somatic gene therapy approaches are not suitable for treating those harboring a germline mutation in a cancercausing gene. In these individuals all cells (at least in some tissues) are at risk for cancer development.

The vast majority of mutations that contribute to cancer are somatic, i.e., present only in the neoplastic cells of the patient. The introduction into cancer cells of a gene that might alter or inhibit the malignant phenotype is an appealing concept. It is based, in part, on experimental data showing that introduction of normal copies of tumor suppressor genes (e.g., p53 or Rb) into cancer cell lines *in vitro* restores normal growth properties.

Daunting hurdles must be overcome if gene correction strategies are to achieve a meaningful clinical outcome. First, some cancers arise following mutations in which the gene product has a dominant effect. Hence, transfer of a normal copy of the gene into an affected cell would have little, if any, impact. Second, the number of cells within a clinically detectable cancer is large (>10^9), and the mutation rate within them is so high that mutations in the introduced gene will arise in at least a subset of cells, inactivating its function and resulting in subsequent reoutgrowth of cancer cells. Third, present technologies allow gene transfer to only a subset of cells within a detectable, local tumor mass. Finally, the major dreaded complication of advanced local cancer is distant metastasis, and current means for transferring DNA do not provide feasible strategies for reaching cells that have spread widely in the body.

Because of these formidable problems, other-more indirect-gene therapy approaches to the treatment of cancer are being considered. Included among these are transfer of genes for cytokines or other immunemodulatory products to cancer cells either outside the body (*ex vivo*) or directly into the patient (*in vivo*) in an attempt to stimulate immune recognition of not only the gene-modified cancer cells but also cancer cells that have not received the gene situated elsewhere in the body. In some instances, tumorinfiltrating lymphocytes or other immune effector cells have also been transduced in an attempt to increase their specificity and/or reactivity against tumor cells. Although several of these strategies show promise in mouse models, none has demonstrated efficacy in humans.

A second general approach to the treatment of localized cancers, including brain and liver tumors,

involves *in vivo* delivery to cancer cells of genes encoding viral or bacterial enzymes involved in the conversion of nontoxic prodrugs to their active molecules. In one approach the thymidine kinase gene from herpes simplex virus into cells is transferred into cells, rendering them more susceptible to the drug ganciclovir. Finally, genes that provide enhanced resistance to conventional chemotherapeutic agents are being transferred into bone marrow cells, which are then used to reconstitute the bone marrow of patients before treatment with intensive, and otherwise lethal, chemotherapeutic regimens.

- **Infectious diseases:** In principle, a number of chronic infectious diseases, including several types of hepatitis and herpesvirus infections, may be suitable targets for gene therapy approaches. However, only HIV infection has received much attention to date. Current efforts focus on two general areas: postexposure vaccination in an attempt to boost the host immune response to the infection and attempts to express genes in target cells that render them unable to be infected or of supporting HIV replication. Although a handful of trials are ongoing at present, they are in very early stages, and no results have been published.

  In vaccination trials, modified HIV genes are introduced directly into infected individuals following *ex vivo* treatment of target CD4 or precursor cells, typically with retroviral vectors that express genes encoding antiviral products. Several such products are being tested: mutant proteins that inhibit virus replication; antisense RNA that blocks translation of HIV gene products or causes destruction of the HIV genome; ribozymes that attack HIV RNA at specific unique sites; "decoy" RNAs that efficiently compete for binding of viral proteins; and singlechain antibodies that prevent key HIV enzymes from functioning. Although these approaches block HIV replication in cell culture systems, serious obstacles to their practical application remain. Most importantly, it is not yet known what cell types to target, much less how they will be isolated, treated, and returned to the patient. Furthermore, it is unknown whether resistant mutants-the major obstacle to successful drug therapy-will also present a serious problem. Nevertheless, the pursuit of gene therapy remains an active area of acquired immunodeficiency syndrome research, and one that also promises to provide important insights into HIV pathogenesis.

The above discussion illustrates the spectrum of diseases and strategies under consideration for somatic gene therapy and is not meant to be comprehensive. Therapeutic success in most cases will rely on effective gene transfer methods and an understanding of the pathogenesis of each disorder.

## Basic science issues in gene therapy

### Gene transfer and expression

Somatic gene therapy entails two critical steps: delivery of the gene to appropriate cells and its subsequent maintenance and expression. In this section we review current capabilities for meeting these needs.

- **Gene transfer:** Somatic gene therapy requires the transfer of DNA into recipient cells, either outside the body (*ex vivo*) or by direct administration (*in vivo*). Preferably, this should be accomplished without adverse reactions from the recipient. Ordinarily, the intent is to transfer a gene into host cells where it will reside for a prolonged period. Although in many instances, successful therapy will entail gene transfer to specific cells or tissues, target specificity will not always be required. For example, suitable "generic" cells (such as fibroblasts or myoblasts) may

serve as "manufacturing plants" to produce proteins that function in the circulation (e.g., hemophilia) or are taken up by other body cells (e.g., in some enzyme storage disorders).

Several different systems are in use or under consideration for somatic gene transfer (see Table 1). These include DNA (either naked or complexed), RNA viruses (retroviruses), and DNA viruses (adenovirus, adenoassociated virus [AAV], herpesvirus, and poxvirus). Experience is more extensive with retroviral vectors than with other viruses or nonviral DNA. Each vector system has perceived advantages and disadvantages which influence their selection for current or projected clinical applications (see Table 1). Unfortunately, none of the available vector systems is entirely satisfactory, and many of the perceived advantages of vector systems have not been experimentally validated. Until progress is made in these areas, slow and erratic success in applying gene transfer methods to patients can be expected.

The basic biology of retroviruses is the best understood of the vector systems used for gene transfer experiments. Accordingly, retroviruses are employed in the majority of clinical protocols (see Table 2). Among their advantages are efficient entry into dividing cells and integration of the transferred genetic material into the host genome without concomitant introduction of viral genes. Retroviruses would appear to be most suitable for permanent correction of genetic diseases. A major disadvantage of retroviruses is that they infect and integrate only dividing cells. Other problems include cumbersome preparation and relatively low titer, size constraints on inserted genes, difficulties in controlling or ensuring expression, and the potential for genetic damage due to random integration in the host genome.

The adenovirus vector system has found advocates more recently. Among its advantages are high titers and levels of expression, relative ease of handling, efficient infection of many types of human cells, and capacity to infect nondividing cells. Major disadvantages include its relatively high immunogenicity and the complexity of its genome. Despite the widespread belief that adenovirus does not integrate into the host genome, experimental evidence for this assertion is lacking. The persistence and expression of adenoviruses *in vivo* in somatic gene therapy situations are under investigation in several laboratories.

Experience with other DNA viral systems is less extensive. A major perceived strength of AAV is integration at a specific site in the infected cell genome, a finding confirmed thus far only for the wildtype virus. Research with AAV and herpesvirus has been impeded by the lack of suitable helper cell lines for preparing large amounts of pure, recombinant virus. Poxviruses appear most suitable for vaccination.

Direct administration of DNA or DNA complexes (e.g., liposomes) *in vivo* is in its infancy. The ease of preparation and virtually unlimited size of constructs for gene delivery make this approach attractive. The lower efficiency of gene transfer (compared with viruses) and the absence of mechanisms for specifically maintaining the introduced DNA within the cell are major disadvantages. However, the use of naked DNA for *in vivo* vaccination appears feasible and highly promising.

Rather than delivering a particular gene to all cells *ex vivo* or to a specific tissue *in vivo*, it appears preferable to target gene transfer to a particular cell type. In principle, this might be accomplished by incorporating ligands for cell surface receptors into viral envelopes or DNA complexes. However, such strategies have not yet reached clinical application.

Of the vector systems studied to date, retroviruses appear to be most suited for delivering genes to host cells in a stable form due to the efficient integration of retrovirally transduced genes. Studies of yeast cells have defined many of the components necessary for maintaining chromosomes within cells. In principle, the development of artificial human chromosomes as vectors might allow for maintenance of transferred genes without the problems resulting from random insertion of foreign sequences into the host genome. Several laboratories are trying to design such vectors. The efficient introduction of these vectors into cells, however, is likely to be a formidable obstacle to their use for gene therapy in the foreseeable future.

- **Expression of transferred genes:** Expression of transferred genes is essential for successful gene therapy. Much is known regarding DNA sequences that direct highlevel, tissuespecific expression of genes in cells in tissue culture or in transgenic mice. In practice, highlevel expression of genes transferred to somatic cells may not persist or be consistently achieved. Whether these difficulties reflect undefined cellular mechanisms that repress virally introduced genes, a subtle selective disadvantage of stem cells expressing transferred genes, or the failure to include appropriate positive regulatory sequences in the constructs is unknown.

  These uncertainties point to the relative dearth of wellcontrolled studies of appropriate and sustained gene expression following somatic gene transfer into animals. In many of the published reports in this field, gene expression was monitored by highly sensitive surrogate methods (e.g., cellular resistance to the drug G418 or reverse-transcriptase PCR assay), rather than by direct measurement of the desired protein product by immunologic or enzymatic activity. This practice reflects the generally low absolute level of gene expression achieved in many instances, leading to a reliance on nonquantitative analyses.

  How have some of these problems of gene transfer and expression been reflected in gene therapy experiments involving animals and human subjects? Studies of retrovirusbased gene transfer into hematopoietic stem cells provide one perspective. In mice, current protocols permit transfer of genes into a substantial fraction of stem cells following retroviral infection of marrow cells *ex vivo*. Nevertheless, gene transfer into marrow stem cells of other species (including humans, other primates, and canines) has been much less efficient, with 10% or fewer cells transduced. In clinical protocols to date, the low efficiency of gene transfer is particularly notable. This inefficiency reduces potential benefits of introducing a particular foreign gene, and interferes with efforts to measure expression *in vivo*. Hence, both the clinical benefit and scientific value of clinical trials are compromised.

  Current data are largely inadequate with respect to experimental study of the expression of transferred genes. In mouse experiments, longterm expression of transferred genes has been reported, but the consistency of achieving such results is unknown. Also, the quantitation of levels of gene expression over time has not received adequate attention. In human trials, the extent of gene expression is uncertain. In many instances, the efficiency of gene transfer is so poor that investigators have relied on highly sensitive molecular methods (such as reverse transcriptase PCR) rather than biologically more meaningful protein assays, to evaluate expression *in vivo*.

- **Appropriate tissue expression and recipient cells:** Gene therapy approaches would be appreciably enhanced by directing gene transfer and/or expression to the appropriate cells of the body. *Ex vivo* approaches help to ensure that gene transfer is limited to cells of a particular organ. For example, gene transfer into bone marrow cells provides a means to introduce genes selectively

into various blood cell types, including hematopoietic stem cells. Providing a gene product to distinct cell types *in vivo* necessitates either targeting of gene transfer to specific cells or selective expression of introduced genes in specific cell types. To approach the former problem, research aims to incorporate ligands for cellular receptors into viral envelopes or achieve cellspecific gene transfer by binding of virus and target cells to particular proteins or fusion proteins. Meanwhile, tissuespecific gene expression of transferred genes may be accomplished by including appropriate regulatory sequences in gene transfer vectors. Some of these regulatory sequences may be responsive to drugs; hence, *in vivo* expression of transferred genes might be regulated by administration of the relevant drug to the host. Research in these areas within the context of gene therapy strategies is in its infancy.

**Disease pathophysiology**

Cloning genes and characterizing mutations responsible for human disorders are but two of the essential steps in understanding disease pathogenesis. Defining the mechanisms by which mutations lead to pathology is important in conceptualizing approaches to therapy. For example, some mutations may abolish gene function; in these situations, replacing the missing protein may provide adequate therapy. Alternatively, mutations may alter protein function so as to inhibit a cellular pathway (a dominantnegative mechanism). In these instances, shuttingoff expression of the mutant protein or interfering with its function might constitute therapy.

A basic understanding of the pathophysiology of disease is therefore highly relevant when designing gene therapy strategies. Besides understanding how a mutation leads to disease, it is important to determine which cells of the body are suitable targets for effective therapy. Disorders resulting from the deficiency of a circulating protein (e.g., clotting factors VIII or IX in hemophilia) might be corrected by expression of the relevant gene in skin or muscle cells, even if the protein is normally made in liver, as long as it is secreted into the bloodstream. In many other situations, expression of a transferred gene is required in a particular tissue. For example, correction of primary hemoglobinopathies, such as sickle cell anemia and Cooley's anemia, necessitates precisely regulated expression of globin chains in developing red blood cell precursors. For cystic fibrosis, which is due to loss or malfunction of a membrane protein (CFTR), it is relevant to ascertain which, and how many, cells of the lung need to express a normal CFTR gene.

Study of disease pathogenesis may sometimes lead to the development of highly effective new therapies, as illustrated by now classic research on the biochemical basis of hypercholesterolemia. Elucidation of feedback regulation of cholesterol biosynthesis led directly to the testing of HMGCoA reductase inhibitors as cholesterol lowering drugs. These agents, which are in use worldwide, have been shown to be effective in preventing cardiovascular disease. In the current climate, where the cloning of a new disease gene is often viewed principally in the context of gene therapy, the discovery of these drugs might not have been made.

**Animal models of disease**

Principles of disease pathogenesis and the development of gene therapy approaches can often be addressed by studying animal models of human disease. Specific hypotheses and experimental therapies should generally be tested extensively in small animals prior to human experiments. The following questions are representative of those that may be profitably addressed in animal experiments. Can particular cell types serve as appropriate targets for gene therapy? Can bone marrow expression of a

gene product whose deficiency leads to a storage d order affecting the brain improve central nervous system function? What fraction of cells of a tissue need to be altered genetically in order to effect clinical improvement? Are gene modified cells at a selective advantage or disadvantage *in vivo*? Does the host develop an immune response to the gene transfer vehicles or to the newly introduced gene product? Animal models can provide an important link in the development of gene therapy approaches, lying between gene discovery and characterization and clinical experiments. Animal models also constitute a valuable resource for testing other forms of therapy that are not based on gene transfer approaches.

Animal models for genetic diseases have arisen spontaneously in a variety of species (e.g., mouse, cat, dog). Using new methods to mutate genes in embryonic stem cells, mice with engineered alterations in any given gene can be produced. Numerous mouse strains with mutations in genes relevant to human diseases have already been created in this manner, and also by injection of human genes into fertilized mouse eggs. In some instances, mice with such mutations exhibit a phenotype similar to that seen in humans (examples: chronic granulomatous disease, hemophilia A, spinocerebellar ataxia1). In others, the effects of specific mutations in the mouse appear more severe than in humans (examples: ADA deficiency, Gaucher's disease).

Unfortunately, however, mouse models often do not faithfully mimic the relevant human conditions. For example, hypoxanthine phosphoribosyltransferase deficiency associated with LeschNyhan disease in humans is benign in mice due to the presence of an alternative metabolic pathway. Mice with mutations in the CFTR gene do not exhibit the pulmonary effects of cystic fibrosis seen in man, but rather suffer from severe gastrointestinal obstruction. Studying the differences between human diseases and animal model phenotypes may provide insights into disease pathogenesis that may, in turn, be exploited either by gene therapy or pharmacological approaches. Animal models for many cancers and for HIV infection have also been developed. In these instances, the relevance of animal models to human disease appears less certain than in typical singlegene disorders.

Despite potential phenotypic differences between human patients and animal models of disease, the study of animal models for the design of gene therapy approaches in a preclinical setting is important and should not be undervalued. As additional genes leading to human diseases are isolated, and gene targeting and transgenic technologies generate more mouse models of various human diseases, we should anticipate an increasingly productive use of such models to elucidate disease pathophysiology, possibly leading to gene therapy approaches.

Confidence in current approaches to somatic gene therapy would rise if a genuine genetic deficiency in an animal were unequivocally corrected. Although genetic defects in animals have been corrected by introducing transgenes into the germline (or by interbreeding with transgenic animals), somatic gene transfer has not permanently corrected a genetic disease in an animal (e.g., a mouse model of a singlegene disorder).

## Recommendations for Basic Science Research:

1. Given the central role of vectors for delivering genes to somatic cells for therapeutic purposes, the Panel endorses *vigorous* and *expanded* research aimed at developing improved vectors. Special emphasis should be placed on the development of viral and nonviral vectors suitable for gene therapy approaches, stable nonintegrating vectors (e.g., artificial chromosomes), vectors capable of efficient gene transfer into nondividing cells, and vectors designed for tissuerestricted targeting and/or regulated

expression.

It is unlikely that a single vector will prove optimal for all gene therapy approaches. We, therefore, urge the NIH to support wideranging research in vector development and allied areas. An understanding of the behavior of vectors and the fate of DNA introduced into somatic cells will require basic efforts in virology, cell biology, immunology, and the chemistry of DNA complexes. These efforts should also include novel approaches to the selective inhibition of gene function including, but not limited to, the continued development of antisense and ribozyme strategies.

2. To facilitate interdisciplinary efforts to develop optimal vectors, the NIH should consider several strategies, including workshops and program announcements, to stimulate discovery, interchange, and collaboration among scientists in diverse areas.

3. The Panel finds that very little research effort is focused on understanding the mechanisms that govern maintenance or shutoff of gene expression following gene delivery in gene therapy experiments. Available data are largely anecdotal. We urge the NIH to give high priority to basic research to elucidate how recipient cells, and particularly stem cells, handle and express foreign DNA sequences.

4. The Panel urges expanded NIH research into the biology of stem cells in diverse organ systems, as such cells are particularly favorable recipients for permanent correction of monogenic disorders. Specific topics include identifying and enriching stem cells from various organs, targeted transfer into and expression of genes in stem cells, the discovery of growth factors required by stem cells, and methods for selectively modifying genes in stem cells.

5. In the enthusiasm to begin human gene therapy trials soon after gene discovery, important aspects of disease pathophysiology, cell biology, and biochemistry have often been underemphasized. Better elucidation of these aspects will reveal the nature of the target cells within a tissue that need to receive the transferred gene, potential difficulties in achieving gene transfer into the appropriate cells or tissue, and features of the relevant protein that may be critical for its function *in vivo*. This increased focus on basic mechanisms of pathophysiology should also foster alternative efforts to develop pharmacological approaches to disease management. We recommend that the NIH vigorously support basic research into molecular mechanisms that produce disease. The present enthusiasm for molecular approaches to therapy, no matter how justified, must not lead to neglect of biochemical and pathophysiologic mechanisms at the tissue and organ level, which may lead to novel therapeutic insights.

6. We recommend that NIH provide continued and expanded support for the development and study of those animal models of disease that faithfully reflect the corresponding human disorders. These models should strengthen the preclinical scientific basis for gene therapy protocols. This approach will often be more costeffective than attempting to perform similar studies in humans.

## Gene therapy in man Status of the field

More than 100 clinical protocols for gene therapy have been reviewed and approved by the RAC and subsequently approved by the NIH Director (Table 3). Indeed, 597 individuals have already undergone gene transfer in experiments involving more than a dozen diseases. The majority of human gene transfer protocols involve some form of cancer, rather than the treatment of inherited disease. A proportion is designed as "gene marking studies" that utilize cells "marked" with an introduced gene to track the cellular origin of tumor recurrence. Retroviruses are employed as gene transfer vehicles in the majority

of protocols (Table 2).

Although widely referred to as "clinical trials," gene transfer protocols to date are in truth smallscale clinical experiments. Such exploratory studies are meant to test the feasibility and safety of administering particular vectors and to evaluate the effects of expressing specific gene products. Because these studies have not been designed to measure efficacy, they do not include sufficient controls to evaluate the true merits of gene therapy or compare this approach with conventional approaches to the same disease.

Only a few of these clinical studies are designed well enough to address fundamental biological questions. Most notable are several elegant gene marking studies investigating the cellular origin of tumor recurrence and other recent studies comparing the relative survival of cells of HIV-patients simultaneously infected with different retroviruses meant to inhibit HIV replication. These well designed studies greatly increase the information that may be extracted from careful clinical experiments involving only a few patients.

Upon reviewing the status of clinical protocols approved for gene transfer the Panel made several observations:

- Efficacy has not been established for any gene therapy protocol. For example, the administration of PEGADA (a preparation of the enzyme adenosine deaminase that is stable *in vivo*) to patients with adenosine deaminase deficiency, though clinically appropriate in light of its demonstrated efficacy, complicates evaluation of patients initially treated with retrovirally transduced lymphocytes and infants more recently treated with transduced cord blood cells. Furthermore, the atypical, rather mild clinical symptoms of some of the first patients before the experimental procedure began complicates any assessment of its effects. In the case of gene transfer for another disorder, treatment results in five patients with homozygous familial hypercholesterolemia were inconsistent and disappointing with only slight or no changes in cholesterol metabolism and levels.

- Adverse short term effects related to gene transfer protocols appear to vary, depending on the nature of the virus used as a vector and the patient to which it is administered. For example, the use of retroviruses in patients with adenosine deaminase deficiency and in marker studies has not been associated with any obvious adverse effects. However, administration of high titer adenovirus vectors to patients with cystic fibrosis has been associated with severe host inflammatory responses.

- Because clinical experience is still so limited, it is not possible to exclude longterm adverse effects of gene transfer therapy, such as might arise from mutations when viral sequences randomly integrate at critical sites in the genome of somatic cells. It must be noted that multiple integration events resulting from repeated administration of large doses of retroviruses theoretically pose a risk for leukemic transformation. Only longitudinal clinical followup of treated patients can provide data on the long term safety of gene therapy protocols.

- Assessment of the results of gene therapy protocols has been hindered in the majority of studies by the low frequency of gene delivery to target cells and the lack of definable biochemical or clinical endpoints.

- Expectations of current gene therapy protocols have been oversold. Overzealous representation of

clinical gene therapy has obscured the exploratory nature of the initial studies colored the manner in which findings are portrayed to the scientific press and public, and led to the widely held, but mistaken, perception that clinical gene therapy is already highly successful. Such misrepresentation threatens confidence in the field and will inevitably lead to disappointment in both medical and lay communities.

Of even greater concern is the possibility that patients, their families, and health providers may make unwise decisions regarding treatment alternatives, holding out for cures that they mistakenly believe are "just around the corner." For instance, patients with cystic fibrosis may be less vigilant about pulmonary management or a couple at risk for producing a child with a lifethreatening genetic disorder may base reproductive decisions on unrealistic expectations of gene therapy. These reallife scenarios illustrate how patients and their families are placed at risk if the information provided to them is overly optimistic regarding the actual development of successful gene therapy.

In view of these and other difficulties, the Panel considered the appropriateness of clinical studies of gene therapy at this time. The consensus view of the Panel is that clinical studies are warranted for several important reasons-precisely those that distinguish basic and clinical investigation:

- It is not always possible to extrapolate results from experiments in animals to human studies. This difficulty is particularly evident with respect to the efficiency of gene delivery and the host response to viral vectors. Although primate experiments might substitute for some human studies, they entail extraordinary costs for meeting animal care needs, and are not entirely adequate for addressing many key issues.

- Animal models are not satisfactory for studying many important human disorders, including cystic fibrosis, various cancers, and AIDS. Therefore, human studies are necessary to develop effective treatments for these and many other diseases.

- Clinical gene therapy studies reveal problems and raise questions that cannot be otherwise anticipated. For example, in the cystic fibrosis studies the magnitude of the host response to adenoviral vectors was underestimated. This realization has directed research efforts toward engineering vectors that cannot express viral gene products and modulating host responses pharmacologically. Such research may have a substantial impact on gene therapy approaches to other diseases.

- Gene therapy clinical research may provide insights into fundamental disease pathology that may direct subsequent treatment approaches. For example, results from gene marking studies permit investigators to design strategies for purging residual cancer cells from the bone marrow of patients. Reciprocal and synergistic relationships between clinical studies and basic research may emerge from initial clinical gene transfer studies.

Many of the issues faced in bringing gene therapy to clinical practice are encountered when any recent discoveries are applied to the management of disease. The success of such endeavors (often termed "translational research") relies on the quality of the underlying science, the care with which clinical protocols are designed, the melding of different disciplines and strategies into a cohesive approach, and the capacity of investigators to bridge science and medicine. Research at the interface of frontier science and patient care is challenging, and requires that investigators have broad training and biological perspective. For this and other fields of clinical investigation to succeed, high standards of experimental design and robust methods for evaluating clinical outcomes are needed. In the Panel's judgment, many

clinical gene therapy studies thus far have not met these standards.

## Recommendations:

1. The Panel insists on the adherence to rigorous standards for what constitutes appropriate and meaningful human experiments or clinical trials. Inadequacies of many clinical studies to date result from insufficient attention to research design, poorly defined molecular and clinical endpoints, and lack of rigor. All studies should define molecular, biochemical, and quantitative clinical endpoints. They also need to address specific hypotheses, enabling investigators to interpret negative as well as positive findings. These standards are no different from those required for other forms of translational clinical research. Relaxed standards are unacceptable and cannot be excused by unbridled enthusiasm for this treatment modality.

2. The Panel endorses efforts to develop broad, interdisciplinary training programs in clinical (or translational) research (see below). Training of clinical investigators with broad experiences in biomedical and clinical activities, including biostatistics, will benefit not only the immediate field of gene therapy, but also other areas of translational research.

3. The Panel urges gene therapy investigators and their sponsors--be they academic, governmental, private, or industrial-to be more circumspect regarding the aims and accomplishments of clinical protocols when discussing their work with the scientific community, the public, and the media.

## Research training and public education

The development of successful gene therapy approaches necessitates involvement of multiple research and clinical disciplines. Few basic scientists are broadly educated regarding the clinical challenges. Similarly, many clinical scientists, and particularly practicing clinicians, are not sufficiently informed regarding the scientific problems faced in gene therapy. As the field of gene therapy expands, the need for appropriately trained professional personnel, including basic scientists with familiarity of disease pathophysiology and medical scientists and physicians with an appreciation of the complex basic science issues, will become even greater.

We cannot predict when the clinical benefits of gene therapy will be realized. The Panel senses that the public has little understanding of the enormous challenges in the field, and may believe its day has already come, or is at least imminent. Raising such false hopes threatens public support, particularly if effective therapies for more common disorders are not quickly delivered, and may encourage patients and their families to make unwise decisions regarding their treatment options. Scientists, clinicians, scientific journalists, and the press need to devote more attention to responsible, public education regarding the current status and prospects for gene therapy.

## Recommendations:

1. The challenging issues faced in clinical applications of gene therapy are common to different areas of medicine. The Panel recommends vigorous support of programs at the postdoctoral level that will combine rigorous training at the interface between clinical and basic science. These programs, which are envisioned to include both M.D. and Ph.D.trained individuals, should not be restricted to the field of gene therapy, but should encompass translational research of all kinds. Mechanisms for physician training in this area could include the use of career development awards based on a program

announcement.

2. The Panel recommends a concerted effort on the part of scientists, clinicians, science writers, research advocates, research institutions, and the press to inform the public regarding not only the great promise of gene therapy but also current realities. This program of education needs to stress that some time will be required to develop the science of the field and to translate these advances to clinical practice.

3. The Panel urges those who care for patients and provide advice regarding treatment and reproductive options to present the current capabilities of the gene therapy field in an honest and restrained manner. Otherwise, patients and their families may fail to utilize more conventional therapies from which they may receive substantial clinical benefit or choose reproductive options based on unrealistic expectations of curative gene therapy.

## Resources

Gene therapy depends on multiple resources for generation of approved vectors for clinical use and for clinical management of treated patients. A perceived impediment to the initiation of clinical protocols is the high expense of producing viral vectors that meet good manufacturing practice (GMP)standards. Production of retrovirus for clinical use costs $100,000 or more, an amount beyond the budget of most laboratories or academic institutions. In most instances, vectors have been prepared on contract, often by industry. In response to requests from the gene therapy community for resources for vector production, the NIH funded three central vector production facilities. These sites represent a modest NIH investment in this area that cannot realistically fulfill all requests for vector production. Uncertainties regarding which vectors may be best suited for specific clinical studies argue against establishing a large national infrastructure for vector production. Instead, the use of the recently funded program should be critically evaluated and assessed periodically. Meanwhile, the vector production sites should pay particular attention to applications requesting vectors for use in protocols that emphasize rigorous experimental design and the testing of hypotheses, rather than those that duplicate efforts of other institutions. Furthermore, the relative costs of vector production at the NIHsupported sites should be carefully compared with those incurred in producing vectors under contracts to industry. Only then will it be possible to determine the value of the NIH's investment in vector production.

Resources currently exist at many institutions for the performance of clinical studies related to gene therapy. The NIH-supported general clinical research centers (GCRCs) represent a highly appropriate resource for the community.

## Recommendations:

1. The Panel does not endorse the expansion of the NIHsupported vector core program at this time. The need for additional resources in this area should be reexamined periodically.

2. For clinical studies, the Panel urges that investigators make efficient use of NIHsupported GCRCs. These centers have been established to promote research at the interface of clinical and laboratory sciences and are well suited for use in human clinical trials.

## Grants and review process

If gene therapy is to develop as a practical and useful treatment option, major improvements in diverse

areas-including vector systems, gene expression following gene transfer, identification and manipulation of stem cells, generation of appropriate animal models of human diseases, and study of disease pathogenesis-are needed. The Panel discussed the relative merits of different strategies for promoting research excellence. Would needs be best served by establishing additional centers for gene therapy, perhaps organized around specific diseases or organ systems? Or should gene therapy research proposals compete more directly with other forms of research for funding? To what extent do grants in the field of gene therapy receive a fair review, especially given the multidisciplinary nature of the studies? In the Panel's judgment, the best way to foster high quality research and innovation is through competitive peer review, rather than by reliance on special support mechanisms.

NIH has already provided the field of gene therapy with an appropriate start by support of gene therapy centers and specific requests for applications (RFAs). The Panel believes that the current level of research support for this area of biomedicine is appropriate at this time, and suggests that funds for future efforts be allocated on the basis of traditional peer review to ensure that current problems in the field are addressed critically. The adequacy of funding for clinical protocols, particularly outside the NIH campus, has been difficult to assess, since a substantial proportion of support is currently provided by industry. We see no indication that clinical applications in the field of gene therapy are being held back by inadequate financial support.

## Recommendations:

1. The Panel endorses efforts to ensure that rigorous peer review of gene therapy is imposed at all levels, from basic research to clinical trials.

2. To guarantee sound review of gene therapy proposals, particularly those which include clinical studies, the Panel urges that membership of NIH study sections be broadened so that they are better able to review both basic and applied aspects of projects. This view is in agreement with the recommendations of the committee chaired by Keith Yamamoto that recently evaluated the peer review system at the NIH.

3. The Panel recommends that gene therapy research compete directly with all other forms of therapeutic research for funding. Because different approaches may lead to successful treatment of disease, it would be unwise to focus only on one approach, such as gene therapy, for special support.

4. The Panel opposes the formation of study sections dedicated to the review of proposals in the area of gene therapy. If high standards are to be met, research in this area needs to compete with that in other fields of biomedical science.

5. The field of gene therapy should be reviewed periodically to assess whether the investment by NIH should be increased or decreased.

6. To stimulate truly innovative research, the Panel recommends that several Institutes of NIH pool funds through the R21 grant program for short pilot projects focused in specific areas, including vector design and expression of transduced genes, animal models of disease, and stem cell biology.

7. Although it did not formally evaluate the role of RAC, in evaluation of clinical protocols, the Panel recognizes the need for continued review of the safety of gene therapy by expert scientists.

## Role of industry in gene therapy research and clinical activities

Industry substantially influences gene therapy. The field includes both small biotechnology firms which have emerged as a result of activities of academic or NIH investigators, and larger biotechnology companies and traditional pharmaceutical corporations. *In toto*, the research support provided by industry exceeds that of NIH. Therefore, industry plays a major role in the area of gene therapy, one that is certain to increase in future years.

Industry has important attributes that recommend its active participation in gene therapy. First, industry is skilled in translational research and the development of drug products. Second, it has significant experience in meeting high manufacturing and quality control standards, and maintains a professional staff dedicated to regulatory and clinical issues. Third, a high level of scientific and technical expertise characterizes modern biotechnology and pharmaceutical companies.

Several companies have ongoing research programs developing improved vectors for gene delivery and better systems for expression of foreign genes. Moreover, industry has been the major supporter of many of the approved clinical protocols. It is axiomatic that success for biotechnology or pharmaceutical companies will be equated with the development of FDAapproved, clinically efficacious gene transfer treatments for disease. Industrial efforts will focus where the perceived use of the product is greatest, and likely to yield high profits. Hence, industry will tend to concentrate on common diseases, such as cancer, rather than rare disorders. This imbalance has not been evident thus far, as some companies are studying rare diseases initially, aiming to demonstrate proof of concept. For example, industry is supporting clinical studies of adenosine deaminase deficiency, Fanconi's anemia, and cystic fibrosis. Once clinical efficacy of gene therapy procedures is demonstrated for specific, infrequent disorders, however, it can be anticipated that market forces will drive industry's involvement toward common diseases for which patient populations are large.

Industry is collaborating with academic institutions across a wide spectrum. On the whole, this involvement is healthy and complements NIHsupported research. For example, industrial partners have prepared GMPgrade vectors for many clinical studies at academic institutions. The development of gene therapy as a clinical activity is threatened, however, by potential conflicts among the demands of good science and the goals of academic researchers, clinicians, industry, and its investors. The field is at risk to the extent that the premature initiation of clinical studies and overzealous, uncritical reports of clinical results are used by industry to promote investment and perceived research dominance. Likewise, if the objectivity and integrity of academic investigators associated with specific companies is undermined as they seek to maintain their industrial ties, the field will be jeopardized. Decisions regarding diseases to be treated need to be made by investigators on scientific rather than financial criteria. Although the problems of conflict of interest in the field of gene therapy do not differ substantially from those encountered in other forms of clinical research, the wide publicity given to clinical gene therapy efforts raises the potential stakes for both academic investigators and those at companies.

For the future development of the field it will be important that issues of proprietary control not limit the development of clinical protocols. The Panel heard several presentations that described logistical difficulties encountered in gaining industrial approval to perform clinical studies in which cytokines and other reagents were to be obtained from several, often competing companies. These obstacles would be reduced if mechanisms were developed to facilitate the dissemination of useful materials for clinical trials.

In the opinion of the Panel, it is premature to assess what impact, if any, the licensing of a broad patent to a single company for *ex vivo* gene therapy will have on the field. The Panel is concerned, however, that broad patents of this kind will ultimately retard implementation of successful gene therapy protocols once they are developed. Additional study of the impact of patents on the development of the field will be necessary.

## Recommendations:

1. The Panel urges the NIH to maintain support for peerreviewed research in gene therapy and clinical trials, particularly in areas that may not receive attention from the industrial sector, such as the development of gene therapy for rare inherited disorders.

2. NIH should encourage collaborative arrangements that complement NIHsupported research. Industry can play an important role in providing GMPgrade vectors for clinical testing and in designing clinical trials that meet rigorous criteria for efficacy and regulatory standards.

3. The Panel urges the NIH to develop and implement mechanisms that would facilitate the distribution and testing of adjunct materials (e.g., cytokines) for use in gene therapy.

## Intramural NIH support of gene therapy

The first human gene transfer experiments were performed at the NIH, and have engendered excitement within the Intramural Program. At present, more than 100 Intramural investigators are engaged in research pertaining to gene therapy. A much higher proportion of the NIH Intramural research budget than the Extramural budget is devoted to gene therapy, according to information provided by the Institute Directors (5% vs. 1% overall). New collaborative arrangements within the Intramural Program are emerging. Clinical protocols addressing several different disorders, including Gaucher's disease, chronic granulomatous disease, Fanconi's anemia, and cancer, have been approved.

In their presentations to the Panel, Institute Directors discussed and spoke highly of research programs in gene therapy. Of these presentations two aspects are noteworthy. First, there appears to be little coordination of research across Institute boundaries, such that duplicative efforts are inevitable. Second, much of the research utilized similar, yet inadequate, vector systems, which were tailored to deliver genes to the tissue of each Institute's interest. In these respects, Intramural research does not differ from that taking place elsewhere. Research of this kind, however, is unlikely to provide innovative advances. Institute Directors should be encouraged to support innovative research approaches, whether they be Intramural or Extramural, in whatever field of endeavor, even if this leads to deemphasis of gene therapy research within an Institute. They should resist the temptation to fill the "portfolio" with research that appears "hot" but may lack a strong scientific basis or likelihood of success relative to other areas.

The Clinical Center of the NIH campus is a superb resource for the execution of clinical investigation at all levels. With a new clinical center, currently under development, the NIH would be assured firstrate facilities well into the next century. The NIH Clinical Center and its staff have proved effective over the years in attracting and maintaining a patient base representing a wide spectrum of diseases, including many rare, inherited disorders. As such, it is an excellent resource, both for the Intramural Program and the country. The recent decline in patient occupancy in the Clinical Center is a cause for concern, which is being appropriately addressed. It is hoped that erosion of the excellent patient resource base of the NIH will not occur, so that clinical investigation in the Intramural branch will not be jeopardized.

## Recommendations:

1. The Panel appreciates that the concentration of talented basic and clinical investigators on the NIH campus provides an extraordinary resource for gene therapy research. However, better coordination and scientific review of gene therapy research throughout the NIH campus is needed. Improved coordination and review will foster research excellence and reduce duplication of effort at a time of budgetary constraints.

2. The Panel urges Institute Directors to include gene therapy within their portfolios only when there are compelling scientific reasons. Accordingly, they should resist pressures to include gene therapy (or any other) research to "round out" their programs or compete with other Institutes. Institute Directors should take the lead, where it seems appropriate, to focus efforts on research in gene discovery, diagnosis or disease pathogenesis and await further developments in vector technology before expanding gene therapy programs.

3. The Panel endorses the efforts of the Director of the Clinical Center to develop strategies to maintain the superb clinical base of the NIH Intramural Program.

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## Selected references for further reading:

**Cystic fibrosis:**

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Blaese, R. M. et al. T Lymphocyte-directed gene therapy for ADASCID: Initial trial results after 4 years. Science 270: 475480, 1995.

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Ulmer, J. B. et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259: 17451749, 1993.

**Recent review articles:**

Friedmann, T. The promise and overpromise of human gene therapy. Gene Ther. 1: 217218, 1994.

Yu, M., Poeschla, E., and Wong-Staal, F. Progress towards gene therapy for HIV infection. Gene Therapy 1: 1326, 1994.

**Table 1. Vector systems in use or under consideration for gene therapy**

| System | Advantages | Disadvantages | Accumulated Experience | Current or Projected Application |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Retrovirus | Efficient entry. Efficient, predictable, and stable integration into host cells. Biology is well understood. Slight immunogenicity*. No viral genes in vector. | Low titer. Limited insert size. Infection limited to dividing cells. Expression difficult to control and stabilize. Potential for genetic damage*. Expensive, complex to prepare and validate. | Extensive | Marker studies. ex vivo treatments, particularly for AIDS and cancer. Vaccines. |
| Adenovirus | Efficient entry into most or all cell types. High titers. High level of expression. (In principle) no integration of DNA*. Can infect stationary cells. | Vectors contain many viral genes. Highly immunogenic, stimulating both B and T cell responses. Unsuitable for stem cells. Factors controlling tropism poorly understood. Generation of replication competent virus. | Moderate | Localized in vivo treatments: cystic fibrosis, muscular dystrophy, cancer. |
| Adeno-Associated Virus | Integration at specific sites*. | Requires replicating adenovirus to grow. No helper cell line. Specific integration probably does not occur in absence of viral genes. Very limited insert size. | Moderate | Similar to adenovirus. |
| Herpesvirus | High titers. Neurotropic*. | Complex construction. No packaging cell lines. | Slight | Neurologic disorders. |
| Poxviruses | High titers. Large insert size. High expression. | Highly immunogenic. Similar to adenovirus and herpesvirus. | Moderate | Localized, transient in vivo treatment. |
| Naked DNA | Easy to prepare in quantity. High level of safety*. Virtually unlimited size. No extraneous genes or proteins to induce immune response. Lack of integration*. | Very inefficient entry, uptake into nucleus. No mechanism for persistence or stability. | Moderate | Topical applications, mechanical and accessible (skin, vascular, pulmonary, endothelial cells). |

| | | | | |
|---|---|---|---|---|
| Facilitated DNA (e.g., liposomes) | Same as DNA. More efficient uptake than DNA. Protected from in vivo Targetable to specific cell types*. | Targeting not yet achieved. No mechanism for persistence or stability. Inefficient entry. | Slight | As for naked DNA. |

* Denotes theoretical advantage or concern, but one that has not yet been adequately tested.

**Table 2. Delivery Vehicle of Clinical Gene Transfer Studies**

| System | # of Protocols | Percentage |
|---|---|---|
| Retrovirus vectors | 76 | 71.7 |
| Adenovirus | 15 | 14.2 |
| Adeno-associated viruses | 1 | 0.9 |
| Cationic liposome complex | 12 | 11.3 |
| Plasmid DNA | 2 | 1.9 |

**Table 3. Categories of Clinical Gene Transfer Protocols**

| Category | Disease/Disorder | # of Protocols | Percentage |
|---|---|---|---|
| Inherited Monogenic Disorders | **Total** | 20 | **18.9** |
| | ADA deficiency | 1 | 0.9 |
| | Alpha-1-antitrypsin | 1 | 0.9 |
| | Chronic granulomatous disease | 1 | 0.9 |
| | Cystic fibrosis | 11 | 10.4 |
| | Familial-hypercholesterolemia | 1 | 0.9 |
| | Fanconi anemia | 1 | 0.9 |
| | Gaucher disease | 3 | 2.8 |
| | Hunter syndrome | 1 | 0.9 |
| Infectious Diseases | **Total** | 8 | **7.5** |
| | Human inmunodeficiency virus-1 | 8 | 7.5 |
| Acquired Disorders | **Total** | 2 | 1.9 |
| | Peripheral artery disease | 1 | 0.9 |
| | Rheumatoid arthritis | 1 | 0.9 |
| Cancer (byapproach) | **Total** Antisense | 51 | **49.1** |
| | Chemoprotection | 2 | 1.9 |
| | Immunotherapy/ex vivo | 4 | 3.8 |
| | Immunotherapy/in vivo | 23 | 21.7 |
| | Pro-drug/HSV-TK/ganciclovir | 7 | 6.6 |
| | | 11 | 10.4 |
| | Tumor suppressor gene | 4 | 3.8 |
| Marking Protocols | | 25 | 23.6 |
| All Studies | | 106 | 100.0 |

Data from Debra J. Wilson, Executive Secretary, Subcommittee on Data Management, Office of Recombinant DNA Activities, NIH

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**Appendix A**

**Panel to Assess the NIH Investment in Research on Gene Therapy**
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**Appendix B**

# REPORT OF THE FIRST MEETING, MAY 15-16, 1995

With Dr. Stuart H. Orkin and Dr. Arno G. Motulsky serving as cochairs, the Panel to Assess the NIH Investment in Research on Gene Therapy convened for its first meeting on May 15-16, 1995, at the National Institutes of Health (NIH), Natcher Building, 9000 Rockville Pike, Bethesda, MD 20892. During the course of the twoday meeting, panel members heard from Dr. Harold Varmus, NIH Director, and more than 20 additional NIH representatives. Dr. Varmus delineated the panel's mandate, and other NIH staff members described current extramural and intramural programs supporting or otherwise affecting research on gene therapy.

## Panel Mandate-Dr. Harold Varmus, NIH Director

Despite many challenges since the first gene transfer experiments were undertaken in microorganisms, biomedical researchers have made considerable progress toward realizing genebased therapies for human disease. Although clinical application of this emerging technology is still in an early phase of development, since 1988 the NIH Recombinant DNA Advisory Committee (RAC) has approved more than 100 protocols that involve tests of gene transfer or putative gene therapy procedures in clinical

settings. Another panel, the Ad Hoc Review Committee of the RAC, which is chaired by Inder Verma of the Salk Institute, is examining how RAC functions in its role as reviewer of proposals to conduct clinical trials involving such gene transfers.

In the aggregate, NIH invests nearly $200 million annually in programs supporting and overseeing gene therapy research. Despite enthusiastic interest and early signs of safety and biological feasibility, however, evidence for therapeutic benefit to patients is meager. Moreover, opinions vary as to what gene delivery systems will prove effective over the long term, and there are unsettled questions as to which diseases are appropriate targets for gene therapy during this phase of its development.

The mandate for the Panel to Assess the NIH Investment in Research on Gene Therapy is to review broadly the gene therapy research enterprise, considering (i) current and proposed investments by NIH centers and institutes in gene therapy and related disciplines, (ii) developments affecting gene therapy in the wider community of academic, government, and industrial laboratories, and (iii) evaluation of the NIH investment in the context of other support for gene therapy research, particularly from the U.S. biotechnology industry and also from outside the United States.

From this comprehensive review, the panel is expected to devise a set of recommendations on NIHsponsored gene therapy research-not a rigid plan-to be presented at the meeting of the Advisory Committee to the Director, NIH, in December 1995. The recommendations are expected to help in NIH budget and program planning for FY 1997 (and, to a limited extent, FY 1996) by addressing specific questions, including the following:

- How should funds and efforts be distributed among areas such as gene delivery system development, gene expression, biology of target cells, pathophysiology, and animal models of disease?

- What diseases and organ system targets should be emphasized during this period of gene therapy's development?

- What funding mechanisms will be most effective to meet specific program needs? What should be the roles of Requests for Applications (RFAs); centers; the NIH intramural program; pilot production facilities for developing and handling genes, vectors, and target cells; and training programs?

- How should NIH deal with policy issues such as patents and licenses, and what are the needs for public and professional education on the science and ethics of gene therapy?

The panel is also encouraged to make additional recommendations on how NIH might coordinate interdisciplinary gene therapy-related activities. For example, should NIH consider setting up a central coordinating office for such research? Moreover, the panel should also examine the impediments to progress in this field. In a broader context, panel members are reminded that the overall NIH budget is not likely to grow but is more likely to stay flat or be reduced in the near future. Hence, if increases in gene therapy research are deemed valuable and necessary, they will necessarily come at the expense of other programs.

## NIH Staff Presentations

More than 20 NIH staff members presented information to the panel describing extramural and intramural programs that support or are otherwise relevant to the conduct of gene therapy research. These presentations ranged widely and included descriptions of major and more modest basic and clinical research programs being supported by several institutes and centers, information about grant and contract support mechanisms that may be applicable to future extramural gene therapy programs, available oncampus facilities and current research programs, plans to support a new vector and gene delivery development program, RAC's procedures for conducting reviews of clinical protocols and its experience developing a database for gene transfer clinical trials now under way, and current U.S. patent and licensing policies affecting research in this field.

The National Heart, Lung, and Blood Institute (NHLBI) ($53 million); the National Cancer Institute (NCI) ($10 million); the National Institute of Allergy and Infectious Diseases (NIAID) ($16 million); and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) support the largest efforts in gene therapy research, with seven other institutes sponsoring smaller programs. In addition, the National Center for Human Genome Research, in cooperation with researchers from several other institutes, is developing basic and clinical research projects strictly as part of its intramural program.

The NIH intramural program, from which the first several clinical protocols to be approved arose, continues to have a strong focus on gene therapy research. The wide variety of projects on the NIH campus to study disparate diseases, particularly rare disorders; specialized facilities, including stateoftheart human stem cell processing and transfer technology; an emphasis on highrisk, lab bench-to-bedside research at the clinical center; a concerted effort to reinvigorate the intramural program that features stringent staff reviews and a new tenure track system; and recently mandated incentives to encourage technology transfer from federal laboratories to the private sector are some of the reasons behind this focus. Recently, some 100 researchers in the intramural program formed a campuswide interest group.

A variety of funding mechanisms is available for supporting gene therapy efforts through the NIH extramural program. Researchers may submit investigatorinitiated grant applications, usually R01s, or prepare applications in response to RFAs, which invite investigators to submit proposals for projects in NIHspecified research areas. Typically, NIH commits funds for RFAs that it issues, and applications receive special reviews. Nonetheless, RFAs allow considerable latitude for researchers at different institutions to establish innovative arrangements and to set up collaborative networks.

In addition, there is a more formal grant mechanism for forming specialized multidisciplinary research centers at single institutions or among several institutions in a "Centers without Walls" program. Besides these grant mechanisms, the extramural program also can designate areas for competitive proposals to do contract research and development projects, usually with very specific targets. Beyond these standard funding measures, the NIH Director now has discretionary authority to transfer 1 percent of NIH funds for a particular fiscal year into research areas of special interest or need.

Additional research resources supported by the extramural program of the National Center for Research Resources (NCRR) are part of a nationwide research infrastructure that already supports some gene therapy research activities and could be tailored or expanded to support additional efforts. For example, 14 of 75 general clinical research centers, most associated with U.S. medical schools, are conducting gene transfer trials. A biotechnology resource center now at Louisiana State University maintains an extensive, everexpanding database for human genemapping studies. There are seven regional primate research centers where gene therapy animal model studies can be conducted. As part of a new resource.

three Institutes (NCI, NHLBI, and NIDDK) will begin supporting in mid1995 one to three national gene vector laboratories, whose establishment is based on a $3.5 million setaside for a joint RFA.

Another important element of NIH's overall involvement in gene therapy research is the role it plays in overseeing policy matters such as the review of clinical protocols. As of May 1995, RAC has recommended approval for 105 human gene transfer protocols, including 77 involving some form of cancer, 19 involving various genetic disorders, and 8 on AIDS. Of this total, 25 are genemarking experiments without any direct therapeutic potential. RAC is now streamlining its review procedures, and full responsibility for several categories of review now resides with FDA.

The NIH Office of Technology Transfer (OTT) serves under a congressional mandate to evaluate research and technology supported by the intramural program and to take appropriate steps to ensure that such intellectual property is further developed. Thus, OTT helps in identifying patentable inventions and filing applications, coordinating the development of cooperative research and development agreements (CRADAs) and material transfer agreements with researchers in industry or at universities, and arranging licensing agreements with industrial partners that seek to develop commercial products. NIH researchers, primarily from NCI and NHLBI, have filed 81 gene therapy-related patent applications (some of them diagnostic developments and others research tools). To date, NIH has completed 22 licenses covering gene therapy-related technologies.

## Panel Deliberations

Panel members began to identify problems to address and their general approach for using the next two panel meetings. In general, the panel agreed to invite a total of 12-15 expert speakers to the two meetings, one to be held in Bethesda, Maryland, in July and the other in San Francisco, California, in August. Speakers will be asked to address a series of specific scientific issues affecting gene therapy research, including gene expression; stem cell biology; viral vector and other gene delivery systems; clinical disorders that are targets for gene therapy approaches, including cancer, AIDS, and inherited diseases; industry involvement; and patenting issues. Although an effort will be made to split the two meetings thematically, with the first emphasizing basic science and the second emphasizing applied issues, other constraints from scheduling on relatively short notice may override that design.

The invited speakers, who may include leading exponents in this field and critics, will be asked to focus generically on an assigned topic, not merely to provide a summary of an individual's particular experiences relevant to the topic. In addition to presenting a stateoftheart summary on the assigned topic, speakers will be asked to outline major problems or challenges relevant to the topic, including infrastructure and administrative matters, and to propose ways of solving some of those problems and encouraging progress in their particular subject areas. Speakers will also be asked to provide the panel with a brief summary of important points they plan to make.

In addition to making a general plan for the panel's next two meetings, panel members began to identify problems to address as they assess the NIH investment in gene therapy research. One issue that the panel will consider, which is not unique to gene therapy research, is how different NIH institutes and centers divide resources between intramural and extramural programs. On average, the intramural program budget is about 11 percent of the overall NIH budget, but there is considerable variation across specific programs and projects. Historically, the first few gene therapy clinical protocols were undertaken by researchers in the intramural program, and there is continued strong interest in pursuing such developments. Is that an appropriate strategy?

This issue is related to a more general question of how institutes and centers coordinate overlapping programs in gene therapy research both across extramural portfolios and in the intramural program. In practical terms, a question for the panel may be framed as follows: Should several institutes and centers focus on a few seemingly tractable genetic disorders, such as cystic fibrosis and Gaucher's disease, simultaneously supporting relatively comparable research approaches? Or should early efforts be directed more broadly and targeted for a much more diverse set of diseases?

Other issues that the panel may consider include the following:

- Should there be a special new study section to deal exclusively with gene therapy research and related scientific issues?
- Should NIH efforts to support gene therapy be scaled back rather than accelerated?
- Are recent RFAs issued for specialized gene vector laboratories and for gene therapy programs for specific disorders appropriate at this time? What other diseases or technologies would be appropriate subjects for RFAs?
- What should be done about closing the information gap between the biomedical research community and the wider group of medical practitioners as well as the general public regarding gene therapy?

## Future Meeting

The second meeting of the Panel to Assess the NIH Investment in Research on Gene Therapy is scheduled for July 13-14, 1995, at NIH, and the third meeting is scheduled for August 17-18, 1995, in San Francisco, California.

**List of Speakers**

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**Henning Birkedal-Hansen, D.D.S., Ph.D.**
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**Francis S. Collins, M.D., Ph.D.**

**John I. Gallin, M.D.**
Director
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**Richard J. Hodes, M.D.**
Director
National Institute on Aging

**Claude Lenfant, M.D.**

Director
National Center for Human Genome Research

**Karl Csaky, M.D.**
Medical Officer
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**Carl Dieffenbach, Ph.D.**
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National Institute of Neurological Disorders and Stroke

**Harold Varmus, M.D.**
Director
National Institutes of Health

**Nelson A. Wivel, M.D.**
Director
Office of Recombinant DNA Activities

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# REPORT OF THE SECOND MEETING, JULY 13-14, 1995

With Dr. Stuart H. Orkin and Dr. Arno G. Motulsky serving as co-chairs, the Panel to Assess the NIH Investment in Research on Gene Therapy convened for its second meeting on July 13-14, 1995, at the National Institutes of Health (NIH), Building 31, 9000 Rockville Pike, Bethesda, Maryland 20892. During the course of the two-day meeting, panel members heard from representatives from the academic community and the biotechnology industry who are developing gene vectors and working on clinical protocols in the field of gene therapy. In addition, the committee heard a presentation outlining the impact of patenting on this field. The members of the committee also met for several hours in a closed session.

## Vectors: Technical Issues

Initially, researchers have concentrated on developing viruses to serve as vectors for experimental gene

transfer and potential gene therapy procedures. Several types of viruses are being studied for this purpose, with most efforts focusing almost exclusively on retroviruses. Several other types of virus, including adenovirus (AV), adeno-associated virus (AAV), herpesvirus, and human immunodeficiency virus (HIV), are currently also being developed or at least considered for this purpose. In addition, some research groups are studying non-viral vectors, such as liposomes, cationic detergents, and other chemical ligands, for complexing and carrying DNA molecules into target cells.

Several experts believe that, eventually, these two separate vector strategies may converge as researchers try to develop synthetic or semi-synthetic vectors that incorporate the useful features of viruses and chemical agents. Meanwhile, although specific strategies to build useful vectors have strong advocates, no particular vector has emerged as a clear front runner. Each approach has its own problems, and most of them also share problems.

For example, except for AAV, these virus vectors integrate randomly, if at all, in the host cell's chromosomes. Moreover, transduction efficiencies for the virus vectors vary widely--in part reflecting their poor ability to integrate into the chromosomes of resting cells. This problem may even affect HIV, despite a widely held notion that it can infect resting cells. Nonetheless, according to Dr. Richard Mulligan, some of the more recently refined retroviral vectors efficiently transduce non-resting target cells, particularly if they carry appropriate LTR sequences and selectable marker genes or, in some cases, specific promoter-enhancer sequences.

Another general problem is that very little research has been done to incorporate externally controllable gene sequences into viral vectors. For instance, regulated beta-globin gene expression is perhaps the most widely studied prototype. However, when this gene is transduced successfully into human cells growing in tissue culture, its expression cannot yet be properly regulated. Some of these difficulties in attaining gene regulation may arise because of the randomness of integration.

In part because gene regulation questions are unanswered, determining the appropriate dosage levels for viral vectors presents another major challenge. For example, according to Dr. Alan Smith, in clinical trials involving patients with cystic fibrosis (CF), there is a concern that the vector and the CFTR gene product it carries may pose problems if they are delivered in too high doses. Because CFTR is ordinarily effective in cells when present at very low levels, low doses of the transferred gene may be required for effectiveness and may be less likely to induce host inflammatory responses.

These considerations raise a more general and potentially serious problem, namely that viral vectors may carry genes--either their own or the particular recombinant genes they are modified to carry--that elicit host immune system responses. This phenomenon might interfere with the efficacy of gene therapy procedures, possibly curtailing long-term expression of transferred genes and prohibiting repeat administration of the therapeutic agent. Other factors, such as counter selection of the transduced cell by immune or other mechanisms and the randomness of integration, may also contribute to apparent low transduction efficiencies and/or short-lived expression of transferred genes.

Dr. Smith said that cationic lipid vectors are being improved and now perform as much as 500-fold more effectively than naked DNA but are still less effective than is the AV vector in rodent model systems. A potential advantage of cationic lipids is that they can be administered repeatedly to rodents. However, at high doses they induce some focal inflammatory responses, albeit without evidence of eliciting antibodies or provoking T cell activation. Dr. Smith speculated that cationic lipids activate macrophage cells.

Additional advantages and problems associated with specific vector candidates:

- **Retroviral Vectors** Although retroviral genes have been extensively modified to ensure that these vectors cannot replicate and are unlikely to recombine, this extensive modification makes them that more difficult to produce. For example, sometimes several packaging cell lines are needed to produce the vectors, and these cell lines are difficult to derive and maintain. Integration of retroviral vectors into the host chromosome is random, and expression levels of the transgene vary and often are unacceptably low.

  In addition, host cell range tends to be narrow, although introduction of genes from other viruses such as vesicular stomatitis virus (VSV) may help in broadening that range. However, the presence of VSV genes may introduce new toxicity problems, leading to damage or killing of the host cell.

- **Adenovirus (AV)** Several research groups are investigating whether systematic removal or modification of AV genes can reduce host flammatory responses when this virus serves as a gene vector.

  Dr. James Wilson said that other approaches to controlling the inflammatory response are being considered, including production of antibodies to block T cell activation, use of agents such as the drug cytoxan to block T cell proliferation, and use of cytokines to reduce or block production of neutralizing antibodies.

  Dr. Thomas Shenk said that several AV genes influence tumor formation in animal model systems and malignant transformation of cultured cells. Thus, AV represents a potential problem when modified versions of the virus are used as vectors, even though AV has not been observed to cause human tumors. He also is studying the molecular and cellular events required for AV to recognize, bind to, and penetrate target cells, and to deliver and integrate the genes it carries to the target cell nucleus.

- **Adeno-Associated Virus (AAV)** AAV, when modified to serve as a vector, lacks certain control sequences and has limited DNA (4.4 kb) carrying capacity, according to Dr. Kenneth Berns. Moreover, he pointed out that the virus is difficult to produce in high titers and needs to be purified in cesium chloride gradients, a laborious procedure. Because AAV integration is site specific, at least in the wild type, there is a question whether repeat dosing with this vector will be possible because follow-up doses may be routinely excluded from the AAV-occupied site on the host chromosome. In some researchers' hands, AAV has a very low transduction efficiency unless AV or AV genes are also present.

## Clinical and Animal Model Studies: Technical Issues

Invited speakers described gene therapy clinical trials involving a range of diseases, including inherited conditions such as adenosine deaminase (ADA) deficiency and cystic fibrosis (CF), a range of malignancies, and AIDS. Some of the justification for conducting clinical trials at this relatively early stage of gene therapy's development is that other well-tried approaches have not yielded satisfactory therapies for treating these usually deadly diseases. Another problem, cited frequently in the case of CF and applicable to several other cases, is that animal model systems are far from perfect, sometimes making results from gene transfer experiments incomplete or misleading.

Yet another set of problems entails uncertainties over the target cells for gene transfe procedures. Dr. Arthur Nienhuis noted that several issues may help to account for low overall gene transfer efficiency in clinical settings. These include the phase of the cell growth cycle that a particular target stem cell may be in, the current unavailability of effective cytokines to regulate that cycle, difficulties in stimulating specific viral receptor production by the cell, and problems in improving the transduction efficiency of target cells. Stimulation with cytokines or, alternatively, the introduction of drug resistance markers and subsequent use of the corresponding drug may provide ways of expanding specific transduced target cell populations. However, Dr. Nienhuis cautioned that such approaches are still at a very early, preclinical stage of development.

Results from clinical trials so far are limited. Relatively few patients have been treated; measures of biological response are often not adequately sensitive, except in cases where host inflammatory responses have been reported; the effects observed seem to be erratic; and the reporting of effects so far has been almost entirely anecdotal, rather than in peer reviewed publications.

According to Dr. Ronald Crystal, AV-delivered CFTR genes may be expressed along airways of CF patients as many as four days after being administered; however, that expression is observed in only a low percentage of the patients treated. According to Dr. James Wilson, in other experiments involving CF patients, expression of the CFTR gene is rare, not stable, but also not toxic. Although sustained expression is attained in knock-out mice, efforts to introduce the CFTR gene in other animal model systems tend to induce immune responses directed to vector (AV) genes.

Clinical results are also variable in the few ADA patients who are partaking in gene transfer experiments, according to Dr. Michael Blaese. One youngster has been infused 11 times over 23 months with her own T cells after they were treated with a retrovirus carrying an ADA gene, and ADA+ T cells have persisted for two years following the eleventh infusion. He said there is one copy of vector per peripheral T cell, and a positive signal for circulating mRNA (earlier, that signal was "intermittent"). A complicating factor is that PEG-ADA is still being administered to the patient, albeit in a low dose that was established before she more than doubled in weight.

The results for a second child under the same treatment regime are more ambiguous but apparently less promising. However, Dr. Blaese said that three other children whose cord blood was treated at birth show persistent expression of the vector after more than 12 months following the procedure. In addition, good expression of the ADA retroviral-delivered gene was obtained in vitro from foreskin cells obtained from two of these patients, suggesting that small skin grafts using modified cells might be an effective alternative means of delivering the corrective ADA (or other) genes.

Results from gene transfer experiments involving AIDS or cancer patients are scanty. For example, in some cases the HIV+ member of an identical twin pair develops positive skin responses following a gene transfer procedure, but whether this change will lead to clinical benefits is not yet known.

Dr. Philip Greenberg also refers to "transient" antiviral effects and "proof of concept" in gene transfer experiments involving modified HIV genes in patients with AIDS.

A wide range of clinical experiments involving patients with a variety of cancers is under way. Dr. Blaese said there is some evidence of efficacy, such as tumor shrinkage in patients with glioblastomas. Some of the protocols call for the gene transfer procedure to induce immune system responses against the tumor, according to Dr. Gary Nabel. In some cases, patients appear to go into long-term remission.

in other cases, the effects are transient. Partial effects are commonplace in cancer treatment, and gene therapy approaches therefore may find acceptance as a useful addition to the therapeutic arsenal.

Dr. Nabel and Dr. John Mendelsohn pointed out that, in gene transfer experiments involving cancer patients, better measures of biological activity are needed. This need is particularly acute in early tests involving patients with advanced disease when other treatments and other clinical abnormalities make assessment of a single experimental procedure exceedingly difficult.

**Responses to the question of whether the field is ready for clinical trials:**

- Dr. Mulligan: Too much of current research is "not worth taking to patients." The field needs "wise people to prune and avoid copy cat" projects.

- Dr. Smith: "We don't know it won't work." Regarding uncertainties about identifying and successfully targeting epithelial stem cells in human airways, he said that treatments would need to be repeated because cells are expected to turn over every 60-80 days. Also, problems have been seen in animal models where the transgene was expressed in excess; transfection is inconsistent in monkeys when high-dose vectors are tested but successful at low doses in cotton rats; and the goal is not specifically to achieve stem cell integration or to "duplicate" nature but to produce a "useful" therapeutic agent.

- Dr. Crystal: Through clinical trials, investigators are "learning how to evaluate" the gene transfer procedures. In the case of trials involving CF patients, currently antibody-based tests are not sensitive enough to detect the product of the transfected CFTR gene; there are other difficulties with PCR-based assays. Non-human primates, such as rhesus monkeys, are not a reliable model for CF.

- Dr. Mendelsohn: Oncologists have taken drug studies as far as seems possible so the "new approach of gene transfer is exciting ... and needs to be backed."

- Dr. Shenk: If gene transfer procedures appear to work in animal models of some diseases, particularly cancer, they are probably ready for clinical trials. For other diseases, such as CF, particular problems with vectors and gene delivery came to light only because of findings from early-stage clinical trials. Sometimes researchers are unaware of a phenomenon until they do clinical trials and would not have known to look for it during animal experiments. Once appreciated, the phenomenon may better be studied in model systems. However, a moratorium on clinical trials is not warranted.

## Basic and Clinical Infrastructure and Training Issues

Speakers identified several areas of basic biology research that need greater emphasis:

- better understanding of hematopoietic cells and of bone marrow transplantation; stem cell heterogeneity; lung epithelial biology; inflammatory responses; and apoptosis, which may prove important for treating diseases such as cancer and AIDS;

- better understanding of basic virology and manipulations needed to improve vectors and their delivery to appropriate cells in target tissues and organs or to tumors; and

- better models for preclinical studies of disorders that may be subject to gene therapy approaches; however non-human primate models cannot replace clinical research because they are difficult to develop and costly to use.

Speakers also identified several logistical and pragmatic barriers to overcome to foster progress in gene therapy research:

- Means are needed for producing high amounts of vectors of suitable quality for use in small-scale clinical experiments; there is disagreement whether NIH should sponsor GMP vector production facilities.

- More sensitive and reliable assays are needed for assessing the biological activity of transferred genes and clinical end points.

- Novel relations among government, industry, and academic institutions will be needed at the research level and as novel, clinically useful reagents are developed; more than 50 companies are said to be doing gene therapy-related research.

- Industry representatives referred to regulatory impediments and criticized the current clinical protocol review process involving oversight by the NIH RAC and FDA.

- Some participants raised the issue of conflicts of interest.

- One speaker suggested that more international collaborations should be encouraged.

Several speakers referred to training needs, but there is not full agreement on the kind of training that should be emphasized. In general, participants said they prefer rigorous training in basic scientific disciplines, even for young clinical investigators who want to work in the field of gene therapy. There is some sense that, if gene therapy develops rapidly into a successful clinical modality, new means will be needed to integrate these approaches into the current system for delivering health care, which itself is rapidly changing.

## Patent Issues

Because many patent applications pertaining to gene therapy technology are still pending, their impact on this emerging field remains difficult to predict, according to Ms. Rebecca Eisenberg. She recommends that research institutions rely more on non-exclusive licensing agreements as a way of circumventing several potential problems and thereby not hindering the efficient development of this field.

The U.S. Patent and Trademark Office (PTO) has issued several broad-based patents covering fundamental gene therapy technologies, including a patent granted to NIH and licensed exclusively to Gene Therapy, Inc., covering ex vivo gene therapy and another patent granted to the University of Michigan and licensed exclusively to Genovo that covers any viral gene therapy vector carrying the CFTR gene, which is impaired in individuals with CF.

Ms. Eisenberg said that these examples as well as other signs indicate this field of biotechnology is likely to be "more littered" with patents than is the earlier emerging field of biotechnology involving the

discovery and development of therapeutic proteins.

Ms. Eisenberg attributes this difference to the fact that universities and other research institutions are being even more aggressive now than a few years ago in pursuing patent protection for intellectual property their researchers are developing. The Bayh-Dole Act, which specifies that such institutions may retain ownership in patents arising from federally sponsored research, now provides strong incentives for pursuing patents--raising expectations in the university community that royalties from licensing agreements eventually will become a significant source of revenue.

Although in some noteworthy cases involving biotechnology inventions universities are benefitting from significant royalty payments, there are potential problems to face from the flurry of patent applications being put forth in the field of gene therapy, according to Ms. Eisenberg. Perhaps chief among them is that research teams and clinicians may, in effect, be faced with a series of "toll booths" along the road to developing and implementing effective gene therapy procedures. She says that research groups may be hemmed in and financially pinched if they have to enter into complex cross-licensing agreements or if institutions set royalty requirements at levels that are too high. Additional complications include potential priority disputes between competing "inventors," disagreements over ownership when researchers at several institutions are collaborating on a project, and differences arising because some researchers such as medical geneticists tend not to patent their work, whereas other researchers such as molecular biologists do so.

## Future Meeting

The third meeting of the Panel to Assess the NIH Investment in Research on Gene Therapy is scheduled for August 17-18, 1995, in San Francisco, California.

**List of Speakers**

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# REPORT OF THE THIRD MEETING, AUGUST 17-18, 1995

With Dr. Stuart H. Orkin and Dr. Arno G. Motulsky serving as co-chairs, the Panel to Assess the NIH Investment in Research on Gene Therapy convened for its third meeting on August 17-18, 1995, at the Sir Francis Drake Hotel, San Francisco, California. During the first day of the two-day meeting, panel members heard from several representatives of the academic community and the biotechnology industry who are developing gene vectors and working on clinical protocols in the field of gene therapy. The panel members also heard from researchers outside this field who are working at a more basic level. Some of these researchers are skeptical about certain developments in gene therapy, calling some of them misguided, others premature. On the second day, the members of the committee met in a closed session to outline the report they plan to deliver to NIH Director Harold Varmus.

## The Case for Re-Emphasizing Basic Research

Several investigators who appeared before the panel made a case for re-emphasizing basic research and pursuing other strategies for treating some of the diseases that researchers in the field of gene therapy have been studying. One line of argument is that alternative biochemical manipulations appear simpler to apply than gene transfer techniques and might reach fruition sooner. Another line of argument is that gene transfer approaches are premature because not enough is understood in the field of stem cell biology, a vital prerequisite for success in gene therapy.

Some of these investigators criticized current proponents of gene therapy for portraying the field in unrealistic terms and misrepresenting progress as more rapid than it has been. For example, Dr. Joseph Goldstein called for greater realism in the way these researchers present views of their field to the public. He also pointed out that the development of any new therapeutic product is a laborious, time-consuming effort.

Dr. Goldstein said that some of the diseases now targeted by gene therapy researchers might be treated sooner, by other strategies, if investigators pursued more traditional studies into the pathophysiologic basis of the diseases in question. He cited several examples where this alternative approach has paid off either recently or several decades ago. For instance, prednisone treatment reverses steps in a defective sterol metabolic pathway that otherwise leads to masculinization. In a more recent development, an inhibitor of cholesterol production (lovastatin) overcomes a LDL receptor deficiency and, by lowering cholesterol levels, helps to prevent coronary heart disease.

Dr. Goldstein also referred to several genetic diseases that arise because of protein trafficking abnormalities. In some of those cases, the critical mutations lie outside the functional coding region of the enzyme product and, instead, serve to misdirect nascent proteins, which are transported into the wrong biological compartments. He called for basic research that could provide an alternative means to gene therapy for correcting such defects.

Dr. Irving Weissman and Dr. Goldstein said that studies with animal models deserve greater emphasis than they are receiving by researchers who are moving quickly from basic research to the clinic to test new ideas about gene therapy. This general problem is particularly applicable to several unsolved problems involving stem cells, which are important but elusive targets of many gene transfer protocols in which long-term gene expression is a major goal.

Dr. Weissman pointed out that stem cell biology in humans and mice is essentially equivalent. From studies on mice, investigators have learned that there are three critical subsets of stem cells in bone marrow and that the most desirable subset for gene transfer is the rarest and is very difficult to work with.

A key problem in the use of retroviral vectors is to determine which factors will induce self-renewing stem cells to divide. Without such detailed information that can be applied practically, gene transfer procedures will likely fail because genes will not be integrating into target progenitor cells. Dr. Weissman said that, with such fundamental obstacles to human gene transfers, it may make sense to focus instead on activating genes that are already present rather than on replacing defective or missing genes.

Dr. Victor Dzau pointed out that, for certain clinical conditions including several that affect the cardiovascular system, short-term rather than long-term gene expression may be all that is needed to address specific problems. Moreover, in a rabbit model system, studies indicate that localized high pressure can improve DNA transduction rates, enabling antisense oligonucleotides to block transiently a cell-proliferative response that otherwise may interfere with surgically grafted blood vessels. Experiments indicate that high pressure also enhances the delivery of oligonucleotides into cultured human cells, improving the efficiency of transduction.

Dr. Gerald Crabtree described the use of synthetic, lipid-soluble dimerizing reagents that can be used to bring cellular regulatory proteins into covalent juxtaposition, thereby changing their functional status. For example, with appropriate dimeric reagents, specific transcriptional factors might be modified in such a way that they permanently activate this process, meaning that a transgenic cell produces high levels of the designated gene product. Another potential use of such dimerizing reagents would be to cross-link specific cell receptors to induce apoptosis. Although this approach shows promise and many other applications are imaginable, studies are limited so far to cellular systems and considerable work will be needed before animal model studies can be undertaken.

## The Case for Simultaneous Basic and Clinical Research

Several investigators who came before the panel said that the rapid movement from the laboratory to the clinic to test gene transfer protocols sometimes is essential. Dr. W. French Anderson said that, with more than 120 clinical protocols now approved, the nearly five-year-old field of gene therapy research is showing healthy progress. He also predicted that it will be 15 to 20 years before the full potential of current research will be realized.

Dr. Flossie Wong-Staal pointed out that in vitro studies or animal models of AIDS are far from adequate, making it best to go forward rapidly with small, focused clinical trials to test gene transfer procedures. Although the rationale for using ribozyme genes to block HIV gene expression appears sound when tested at the cellular level, many questions, such as the extent to which target cells in patients will be genetically modified and then selected and whether HIV will develop resistance to the ribozyme, can only be addressed through clinical studies.

Dr. Anderson outlined a variety of gene therapy research studies at his institution, suggesting that this locally concentrated diversity of interests and ideas is another sign that this field is healthy and populated with creative young investigators. He also described a long-term project that involves making a series of improvements in a current retroviral-based vector that could extend its half-life in the host circulatory system, increase its efficiency of binding to and entering specific target cells of the host, improve its chances of delivering genes for long-term expression, and eventually lead to a readily injectable gene-delivery product. Efforts to realize these goals are only at the "very beginning."

Other current basic research developments may eventually help solve some of the challenges that investigators conducting human gene transfer protocols now face. For example, Dr. Donald Kohn described efforts to modify the long terminal repeat (LTR) in a retroviral vector now being used in gene transfer protocols as a way of extending the expression of transferred genes after they are delivered to target cells. Hematopoietic cells from mice are providing a valuable model in which to study this problem, and some results indicate that methylation within the LTR correlates with the disappearance of transferred gene expression.

In a model system in which human bone marrow cells are introduced into immunologically deficient nude mice, Dr. Kohn and his collaborators find that the addition of stroma enhances gene transfer in vitro and also extends long-term expression of the transduced genes. The impact of growth factors on these steps is also being evaluated. Dr. Kohn said that, despite the value of this information from experiments in mice, clinical trials are needed to understand in detail how each of these steps work in humans.

One important problem that has come to light from early gene transfer clinical studies is that host immune responses may abbreviate expression of transferred genes. Dr. Paul Tolstoshev described efforts to develop sophisticated vectors that can overcome this problem. Less immunogenic vectors are being constructed for use in conjunction with immunosuppressive agents such as dexamethasone or cyclosporin that can reduce immune system responses, including deleterious inflammatory reactions.

## Academic, Industry Representatives' Comments on Policy Questions

Industry and academic representatives said that clinical trials are an important element of gene therapy, providing data that have helped in choosing among models and in other ways are proving essential for the development of this field. Dr. Wong-Staal said that the cost as well as the complexity of current regulatory requirements impose barriers on efforts to design and conduct small-scale clinical trials. Moreover, simplifying annual reporting requirements would be helpful to investigators.

Dr. Anderson pointed out that progress is more likely to be rapid if individual investigators--rather than a central committee--direct research decision making. He also recommended that the development and use of vectors made in NIH-supported specialized laboratories not be restricted to only those researchers whose work is being supported by NIH. He was less certain whether a policy of limiting such vector

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# The mode of presentation and route of administration are critical for the induction of immune responses to p53 and antitumor immunity

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*We have examined the immune response to full-length wild-type human p53 presented by a recombinant canarypox vector (ALVAC) and by plasmid DNA. For the ALVAC recombinant, intravenous, but not subcutaneous, intramuscular or intradermal administration, induced CD8+ CTLs that lysed tumor cells transfected with human mutant p53. Intrasplenic administration also induced CTLs. Biodistribution studies showed that intravenously injected ALVAC localized primarily in the lung, liver and spleen, whereas intramuscularly injected virus remained predominantly at the injection site. Intradermal and intramuscular immunization with naked plasmid DNA encoding human wild-type p53 also induced a specific CTL response. DNA immunization induced complete protection against challenge with a mouse embryo fibroblast transfected with human mutant p53 and partial, but significant, protection against a transfected mastocytoma. The ALVAC recombinant induced partial protection in both models. These results suggest that recombinant ALVAC and DNA might be interesting presentation platforms for p53 to be tested in clinical studies.* © *1997 Published by Elsevier Science Ltd. All rights reserved*

**Keywords:** tumor-associated antigen; vaccination; CTL

In pursuing cancer immunotherapy, it will be important to assess multiple approaches to induce optimal immune responses to tumor-associated antigens (TAAs). In particular, when the target is an intracellular protein, it will be critical to present TAAs in ways that optimize the cellular immune response. Thus, vectors capable of eliciting strong cellular immune responses are of great interest for cancer immunotherapy. Among these are recombinant poxviruses and plasmid DNA. The attenuated canary pox virus ALVAC has been shown in a number of clinical studies to be well tolerated and capable of inducing both humoral and cellular responses[1-3]. Similarly, DNA immunization has been reported to induce humoral and cellular responses and protection against challenge in both mice and chimpanzees[4,5]. These two vectors have also been used successfully to immunize mice against model TAAs[6-10]. However, there have been few direct comparisons of these two presentation platforms. Furthermore, whereas a number of different routes of administration have been used for both pox viruses and DNA, few, if any, studies have systematically compared different routes with a defined antigen.

The product of the p53 tumor suppressor gene is an attractive antigen for cancer immunotherapy, since it is expressed at very low levels in normal cells, but is overexpressed in about 50% of all human cancers due to missense mutations that significantly increase the half-life of the protein[11-13]. Despite the presence of mutations, the immune response in cancer patients appears to be directed largely against wild-type epitopes. The antibodies found in a subset of patients with a variety of different types of p53-overexpressing cancers predominantly recognize non-mutated regions of the protein[14-16]. T cells from a few of these patients have been reported to proliferate in response to wild-type p53[17]. CTLs induced *in vitro* from healthy donor PBLs recognize both wild-type and mutant peptides[18-20] and a CTL clone that recognized a wild-type peptide lysed MHC-matched human tumor cells with p53 mutations[21]. Finally, CTL lines derived from HLA-A2 transgenic mice lysed a number of HLA-A2+, p53 overexpressing breast and colon carcinoma lines[22].

In mice, CTLs specific for mutant p53 have been induced using mutant peptides[23,24] or DNA encoding a

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mutant epitope or the full-length mutant gene[25]. CTLs have also been induced with wild-type peptides[26,27], although the affinity of the CTLs was lower than those induced by mutant peptides[27]. Together with the findings in humans, these results imply that the immune system can recognize self epitopes as well as mutant neoepitopes. Mice immunized with dendritic cells pulsed with a wild-type p53 peptide were protected against a tumor challenge[26]. However, clinical application of this technique would necessitate harvesting dendritic cells from each patient and expanding them *in vitro*, as well as determining the optimal peptide for each MHC haplotype.

An alternative approach is to immunize with the full-length wild-type protein. Recently, Roth *et al.* reported that vaccination with ALVAC encoding wild-type p53 induced protection in a mouse model[7]. However, whereas they saw an antibody response following immunization, they did not mention a CTL response. Since p53 is an intracellular protein, it is unclear how protection was mediated in those experiments. We have extended these studies and shown that the ALVAC recombinant can induce a CTL response, but that the route of administration is critical. We also have induced CTLs by immunizing with a DNA plasmid. However, whereas the ALVAC recombinant induced only partial protection against challenge with p53-transfected tumor lines, DNA induced complete protection in one model system.

## MATERIALS AND METHODS

### Plasmid and virus constructs

The ALVAC recombinant expressing human wild-type p53 (vCP207) has been described previously[7]. vP1101, a NYVAC (attenuated vaccinia virus) recombinant[28] expressing human wild-type p53, vCP297, an ALVAC recombinant expressing *Photinus pyralis* luciferase, and vCP319, an ALVAC recombinant expressing murine granulocyte macrophage colony stimulating factor (GM-CSF), were all produced at Virogenetics, Troy, NY. Two plasmids expressing human wild-type p53 (pC53-$SN_3$) and human p53 with an R to H mutation at amino acid 273 (pC53-4.2$N_3$), both under the control of the human CMV immediate early promoter/enhancer, were the kind gift of Arnold Levine, Princeton University. Both plasmids also contain the $neo^R$ marker allowing selection with G418. The plasmid pMP1406hp53, which also expresses p53 under the control of the human CMV immediate early promoter/enhancer but lacks the $neo^R$ gene, was made by Marion Perkus at Virogenetics.

### Transformed cell lines

P815, a murine mastocytoma line of DBA/2 origin, was a gift from Dr B. Autran, Hôpital Cochin, Paris, France. P815 HFR was another isolate of this line from Emanuelle Trannoy (Pasteur Mérieux Connaught, France). BALB/3T12-3, a tumorigenic fibroblast line derived from BALB/c mouse embryos, was obtained from the ATCC (no. CCL 164).

Cells were transfected with pC53-4.2$N_3$ using LIPOFECTAMINE (GIBCO BRL) for P815 and $CaPO_4$ for BALB/3T12-3. Transfectants were selected and expanded in the presence of 400 μg/ml of G418, and clones that stably expressed p53 were chosen. Clone 2E4 was derived from P815, clone 4D5 from P815 HFR and clone 2-21 from BALB/3T12-3.

### Immunization of mice

Eight-week-old BALB/c ByJ (IFFA-CREDO or Jackson Laboratories) or DBA/2 female mice (IFFA-CREDO) were immunized using a syringe for intravenous (100 μl in the tail vein) and subcutaneous or intramuscular administration (100 μl). For direct intrasplenic administration, mice were anesthetized and shaved, and 20 μl were delivered through the abdominal wall into the spleen. For intradermal administration, mice were anesthetized and shaved, and a Mesoflash apparatus (Prolitech) was used to deliver 20 μl at each of five separate sites on the back. DNA for injection was purified by alkaline lysis followed by CsCl ethidium bromide equilibrium gradient centrifugation or Qiagen column chromatography. Murine GM-CSF was from Peprotech ($10^7$ U/mg protein), and murine interleukin-12 (IL-12) was from R & D Systems.

### ELISA for detection of mouse serum antibodies against human p53

The coating antigen was a nuclear extract of Sf9 insect cells infected with a recombinant baculovirus expressing human wild-type p53 kindly provided by Laurent DeBussche of RPR-GenCell, Vitry-sur-Seine, France[29]. The p53 content of this extract was approximately 50% by electrophoresis. Microtiter plates (96-well) (Dynatech) were coated overnight at 4°C with 100 ng of p53 in 100 μl/well. After washing with PBS-0.05% Tween 20 (PBS–T), plates were blocked for 1 h at 37°C with PBS-T containing 1% bovine serum albumin (PBS–T–BSA) and washed again. Serum, or as a positive control, monoclonal antibody DO1 (Santa Cruz), which recognizes human p53, was added, and plates were incubated for 1.5 h at 37°C. After washing, the secondary antibody, rabbit anti-mouse IgG, IgA, IgM (Zymed) was added at a dilution of 1/1000 in PBS–T–BSA, and incubation was continued for an additional 1.5 h at 37°C. After a final wash, the substrate disodium *p*-nitro-phenyl-phosphate was added at 1 mg/ml in 1 M diethanolamine, 0.3 mM $MgCl_2$, pH 9.8, and plates were developed for 30 min at room temperature in the dark. The reaction was stopped with 50 μl/well 2 N NaOH, and plates were read at 405 nm, using a Molecular Devices plate-reader.

### Determination of specific CTLs against human p53

Spleens from immunized mice were dissociated in RPMI with 2% fetal calf serum (FCS), and splenocytes were prepared. Stimulators (from unimmunized mice; prepared as above) were infected at a multiplicity of infection of 2–10 with vP1101 or vCP207, as indicated. After 1 h at 37°C with 5% $CO_2$, the infected stimulators were washed and irradiated with 25 Gy (or in some experiments were used without irradiation) and mixed with effectors at a 1:2 or 1:5 ratio in a 75 $cm^2$

flask at a final concentration of $2 \times 10^6$ cells/ml. Flasks were incubated vertically for 5 days in a 37°C incubator with 5% $CO_2$. If a second stimulation was performed, a stimulator:effector ratio of 2:1 was used, and 10 U/ml murine interleukin-2 (IL-2; Genzyme) were added. The restimulation was done for 5 days.

For the CTL assay, $10^6$ P815 or 2E4 cells were labelled with 200 $\mu$Ci of $Na_2^{51}CrO_4$ (NEN, 40 mCi/ml) for 1 h at 37°C and washed three times in RPMI with 10% FCS. $5 \times 10^3$–$10^4$ target cells were dispensed into each well of a 96-well round-bottom microtiter dish. Effectors were added, and plates were incubated for 4 h at 37°, after which the supernatant was harvested and counted. Percentage specific lysis was calculated as 100 × (effector release-spontaneous release)/(total release-spontaneous release).

### Depletion of CD4$^+$ and CD8$^+$ T cells

Dynabeads M450 were coated with anti-murine CD4, anti-murine CD8 or, as a control anti-human CD19 and washed twice with PBS containing 10% FCS. Effector cells ($1.2 \times 10^7$ total) were suspended in 2 ml of RPMI and added to the beads at a ratio of one effector cell/two beads. The samples were incubated for 20 min at room temperature, and then the beads were collected for 4 min, using a Dynal MPC magnetic apparatus. The depleted supernatant was collected, and a second depletion was performed, following which, the effectors were used in a Cr-release test, as described above.

### Biodistribution of ALVAC

Male outbred mice, OF1 strain (IFFA-CREDO), were injected intravenously in the tail vein or into the gastrocniemial muscle with the indicated $TCID_{50}$ of vCP297 in a volume of 100 $\mu$l. Organs were removed 6 h later and weighed, and 1 ml of lysis buffer (Promega) was added to each sample, except for the liver where 2 ml of buffer were added. Samples were frozen and stored at −70°C and thawed on the day of luciferase assay and homogenized in a glass rod potter homogenizer. The extracts were centrifuged for 15 min at 10000*g* in a bench top centrifuge, and the supernatants were collected and kept at 4°C until enzymatic assay. Luciferase activity was measured automatically using a Lumat LB9501 luminometer (Berthold) using conditions provided with the luciferase substrate and reaction buffer (Promega). The relative light units (RLU) counted by the luminometer were converted to luciferase weight by comparison of the enzyme activity measured in tissue extracts with the specific activity of a purified preparation of *Photinus pyralis* luciferase EC 1.13.12.7 (Boehringer). The specific activity of the purified enzyme was 3500 RLU per picogram of enzyme, on average. The results were expressed as the weight of luciferase per organ.

### Tumor challenges

Mice were challenged as described in the Results. Animals were examined weekly until tumors were detected, and thereafter, the tumor diameter was measured twice weekly. Tumor-free survival curves were analyzed for statistical significance by the log-rank test of Mantel and Haenszel.

## RESULTS

### Immune response to an ALVAC recombinant encoding human wild-type p53 delivered by different routes

In our initial experiments trying to induce an optimal immune response to p53, we focused on vCP207, a recombinant canary pox (ALVAC) expressing human wild-type p53. Intravenous administration at weeks 0, 3 and 6 of $10^7$ $TCID_{50}$ of the recombinant, but not the parental ALVAC, induced a significant specific IgG response in 11/12 mice. However, when the same vaccination was performed by the subcutaneous route, only three out of 12 mice responded, and even these had titers only marginally above background. We also saw very little response by the intradermal route, which has been used classically for immunization with the prototypical replication-competent poxvirus, vaccinia. Because GM-CSF has been shown to activate and recruit professional antigen presenting cells[30,31], we postulated that it might enhance the response by the intradermal route. However, neither co-administration of $10^6$ $TCID_{50}$ of an ALVAC recombinant expressing murine GM-CSF (vCP319), nor of co-administration of 500 or 5000 units of recombinant murine GM-CSF had any measurable effect on the antibody response (data not shown).

We also examined the specific CTL activity of splenocytes from immunized mice after *in-vitro* stimulation with naïve splenocytes infected with a recombinant highly attenuated vaccinia, NYVAC, expressing human wild-type p53 (vP1101). Splenocytes from mice immunized three times intravenously with vCP207 showed high levels of lysis of clone 2E4, a P815 line transfected with human R273H mutant p53 [*Figure 1*(A)]. There was clear lytic activity, even after one round of *in-vitro* stimulation, although the levels increased, especially at low E:T ratios, after a second round of *in-vitro* stimulation [compare *Figure 1*(A) and (B)]. The response was specific for p53, since we saw only low levels of lysis of 2E4 after immunization with the ALVAC vector and only low levels of lysis of untransfected P815. In contrast to the results by the intravenous route, splenocytes from mice immunized subcutaneously with the same vCP207 showed no evidence of CTL activity, even after two rounds of *in-vitro* stimulation. Intradermal administration also failed to elicit any specific anti-p53 CTLs [*Figure 1*(A)] and addition of GM-CSF in the form of recombinant protein (500 or 5000 units) or vCP319 had no effect (data not shown).

In another experiment, we examined two additional routes of administration [*Figure 1*(B)]. The intramuscular route, like the subcutaneous, failed to induce specific CTLs. Even when we administered 0.5 $\mu$g of murine IL-12 intraperitoneally every day for 5 days beginning on the day after each intramuscular injection, a regimen reported to substantially increase the CTL response to a plasmid-encoded antigen[10], we did not find any CTLs (data not shown). However, direct intrasplenic administration, which has recently been reported to be extremely potent for inducing cellular responses to transfected fibroblasts[32], did induce CTLs.

although it was not clearly superior to the intravenous route. Finally, we considered that the route of administration might be critical only for priming; however, mice primed by the intravenous route and boosted subcutaneously failed to respond [*Figure 1*(B)].

The CTLs induced by intravenous administration of vCP207 appear to be of the $CD8^+$ phenotype. Lysis was completely abrogated by pre-treating effectors with magnetic beads coated with an antibody against murine CD8, whereas neither an antibody against murine CD4 nor a control antibody against human CD19 had any significant effect on CTL lysis (*Figure 2*).

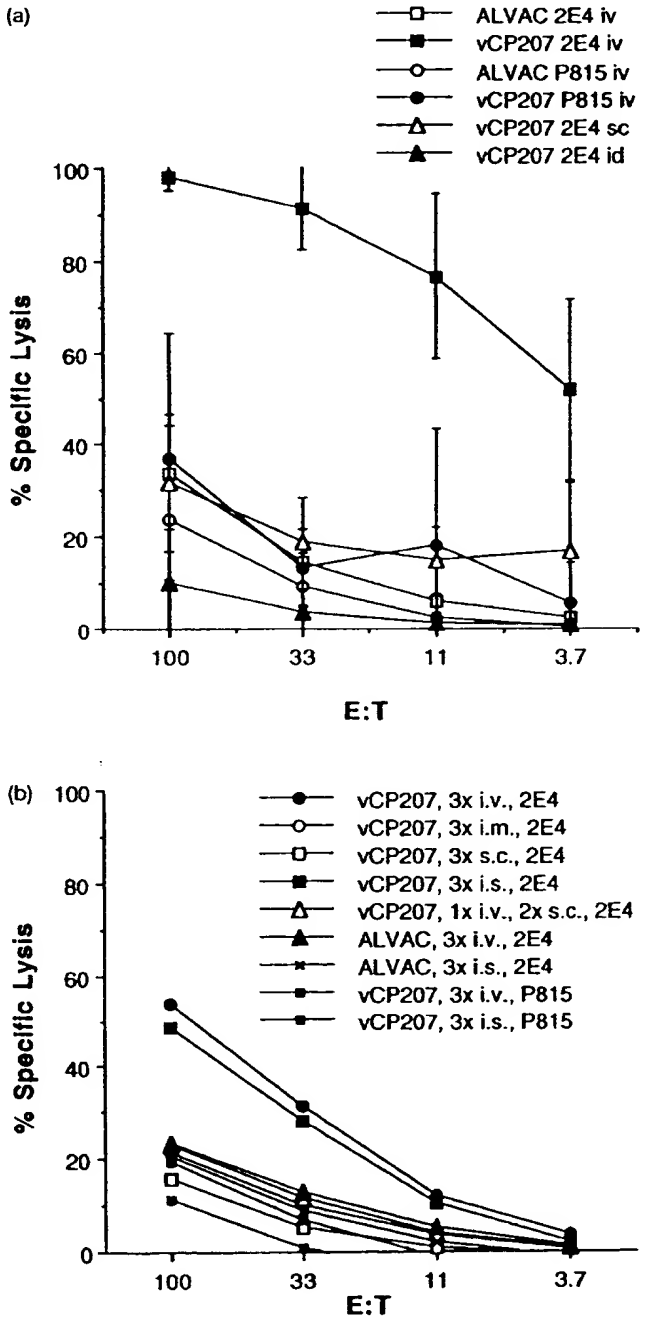


**Figure 1** Anti-p53 CTL Response to vCP207 administered by different routes. Mice were immunized by the indicated routes, at 0, 3 and 6 weeks with $10^7$ $TCID_{50}$ (A) or $5 \times 10^7$ plaque forming units (B) of either vCP207 or parental ALVAC. Two weeks after the last immunization, splenocytes were harvested and stimulated twice (A) or once (B) *in vitro* with naïve splenocytes infected by vP1101. Lysis was measured against parental P815 and 2E4, as indicated. Values in (A) are the mean of four groups of two mice with standard deviations represented by the error bars, whereas values in (B) are from a single pool of four mice

### Biodistribution of ALVAC injected intravenously

In order to try to understand why intravenous administration of vCP207 induced an immune response that was significantly better than that seen with other routes, we analysed the biodistribution profile of ALVAC in mice. In order to maximize our ability to detect virus in different organs, we used a recombinant ALVAC expressing *Photinus pyralis* luciferase, vCP297. Six hours after injection, which we determined to be the time of maximum expression, we assayed the luciferase activity in extracts from a number of different tissues. When $10^6$ or $10^7$ $TCID_{50}$ are administered into the tail vein of mice, most of the virus localizes to the lung, spleen and liver (*Table 1*). A minor fraction remains in the tail at the site of injection, and additional small quantities are found in the plasma and kidneys. By contrast, when the virus is injected locally in the gastrocnemial muscle, virtually all of it remains at the site of injection (*Table 1*).

### Immune response to naked DNA encoding human wild-type p53

We have also examined the response to immunization with a plasmid encoding human wild-type p53 under the control of the CMV promoter (pC53-$SN_3$).

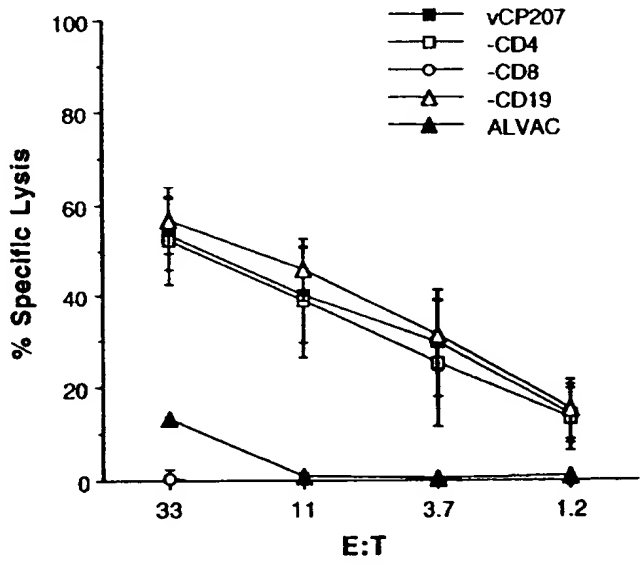


**Figure 2** $CD8^+$ effectors are responsible for the anti-p53 CTLs induced by intravenous administration of vCP207. Mice were immunized intravenously at 0, 3 and 6 weeks with $10^7$ $TCID_{50}$ of vCP207. Splenocytes were harvested 2 weeks after the last injection and stimulated twice *in vitro*. Undepleted effector cells and effector cells depleted with magnetic beads coated with anti-murine CD8, anti-murine CD4 or anti-human CD19, as indicated, were tested for chromium release against 2E4. Undepleted effector cells from mice immunized with ALVAC were also tested. Values are the mean of three groups of two mice with standard deviations represented by the error bars, except for the ALVAC-immunized mice, which is the mean of two groups

**Table 1** Distribution of ALVAC luciferase after intravenous and intramuscular administration

| Administration | Organ | pg luciferase/organ |
|---|---|---|
| Intravenous | Spleen | 189 |
| $10^7$ $TCID_{50}$ | Liver | 204 |
| | Lung | 3194 |
| | Kidneys | 6 |
| | Plasma | 4 |
| | Tail | 10 |
| | Gastrocnemial muscle | 0 |
| | Brain | 0 |
| | Salivary glands | 1 |
| Intravenous | Spleen | 34 |
| $10^6$ $TCID_{50}$ | Liver | 12 |
| | Lung | 46 |
| | Kidneys | 0 |
| | Plasma | 0 |
| | Tail | 2 |
| | Gastrocnemial muscle | 0 |
| | Brain | 0 |
| | Salivary glands | 0 |
| Intramuscular | Gastrocnemial muscle | 122 |
| $10^6$ $TCID_{50}$ | Spleen | 0.1 |
| | Inguinal+iliac lymph nodes | 0.4 |
| | Popliteal lymph node | 0.3 |

Intradermal administration of 100 $\mu$g of this plasmid at weeks 0, 3 and 6 induced a specific antibody response in three out of eight mice, compared to none of the mice for the pUCneo vector. Despite this rather variable antibody response, we saw a CTL response in all groups of animals immunized with 100 $\mu$g, but not 1 or 10 $\mu$g of pC53-$SN_3$ [*Figure 3*(A)]. Like those induced by vCP207, the CTLs induced by p53 DNA were specific for p53, showing no lysis of 2E4 following immunization with the pUCneo vector DNA, nor any significant lysis of untransfected P815. We also saw a clear CTL response after intramuscular injection of 100 $\mu$g of another DNA encoding human wild-type p53, pMP1406hp53 [*Figure 3*(B)].

### Protection against challenge with human p53-transfected cell lines

Once we had determined optimal ways to induce anti-p53 immune responses, we tested whether they would be protective against challenge with murine tumor lines transfected with human p53. In these experiments, we immunized at weeks 0, 3 and 6 and challenged at week 8. One challenge line, clone 2-21, was derived by transfecting BALB/3T12-3, a tumorigenic isolate of BALB/c 3T3, with a plasmid encoding human mutant p53 (R273H). Control immunized BALB/c mice developed tumors, which grew progressively, starting about 6 weeks after subcutaneous injection of $10^7$ cells [*Figure 4*(A)]. Prior intravenous immunization with vCP207 conferred partial, but statistically non-significant ($P > 0.25$ vs. PBS) protection, with only one out of nine mice remaining tumor-free by week 15. This effect was specific for p53, since immunization with the ALVAC vector was not protective, and, in fact, appeared to slightly accelerate tumor growth relative to PBS. Also, vCP207 conferred no protection at all against the untransfected parent BALB/3T12-3 [*Figure 4*(B)]. By contrast to the partial protection induced by the recombinant virus, intradermal immunization with pC53-$SN_3$ DNA induced complete protection [$P < 0.001$ vs. both PBS and pUCneo; *Figure 4*(C)].

Another challenge model, clone 4D5, which was derived by transfecting P815 mastocytoma cells with human R273H mutant p53, forms tumors that appear about 3 weeks after subcutaneous injection of $10^4$ cells in DBA2 mice [*Figure 4*(D)] and grow rapidly, resulting in death by about week 6. In this model, both vCP207 and pC53-$SN_3$ induced partial protection [*Figure 4*(D)], but only the DNA was significantly different from the PBS control group ($P < 0.1$ for vCP207 and $P < 0.01$ for

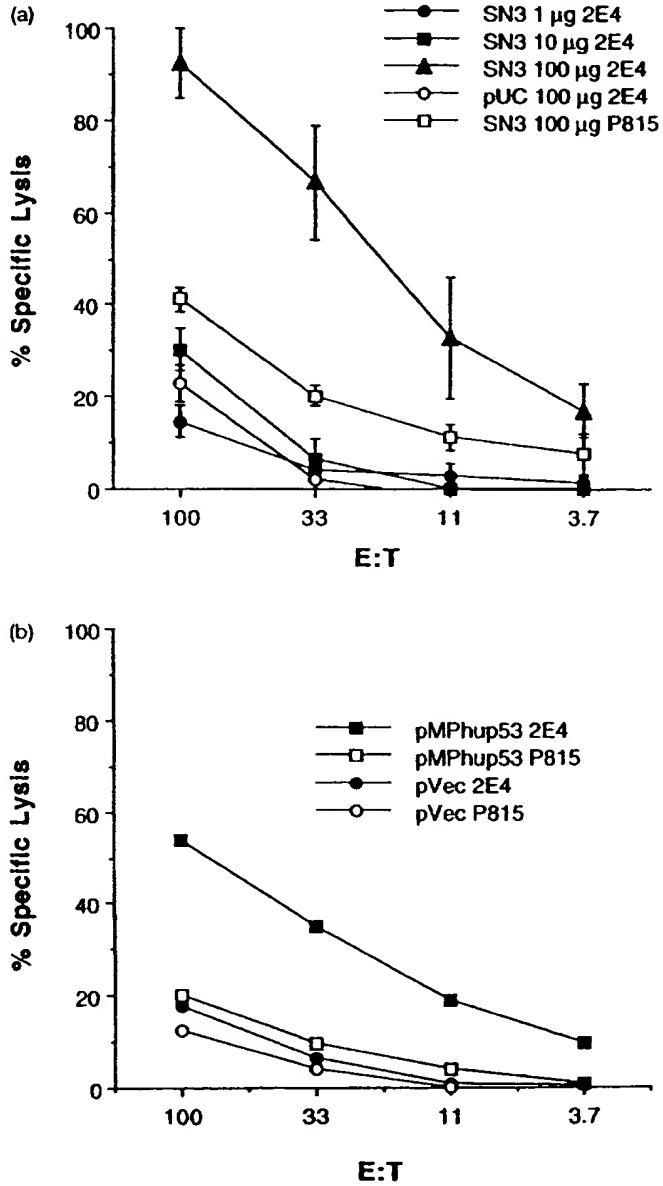


**Figure 3** Anti-p53 immune response induced by intradermal administration of DNA. Mice were immunized intradermally at 0, 3 and 6 weeks with pC53-$SN_3$, or the control plasmid, pUCneo (A), or intramuscularly at 0, 3 and 6 weeks with pMP1406hup53, or vector lacking the p53 gene, pVec(B). The anti-p53 CTL response 2 weeks after the last immunization was measured in splenocytes stimulated twice *in vitro* with naïve splenocytes infected with vCP207 (A), or stimulated once *in vitro* with naïve splenocytes infected with vP1101 (B). Lysis was measured against P815 and 2E4. Values in (A) are the mean of two groups of two mice with standard deviations represented by the error bars, whereas those in (B) are from a single pool of two mice

pC53-SN$_3$). Neither the control ALVAC nor the control DNA induced a significant protection relative to PBS ($P > 0.25$).

## DISCUSSION

We have compared the immune responses induced by delivering the full-length wild-type p53 gene, either via a recombinant viral vector or plasmid DNA. In the case of the recombinant ALVAC, the route of administration was critical for the induction of an optimal immune response. We saw good responses immunizing by the intravenous route, as others have reported for fowlpox[6]. However, the antibody response was significantly weaker by the subcutaneous, intramuscular and intradermal routes, and these routes did not induce any detectable CTLs, even when GM-CSF or IL-12 were co-administered. Since we presume that targeting to the spleen, which is rich in APCs and cytokines, is at least in part responsible for the immunogenicity of the intravenous route, it is interesting that direct intrasplenic administration also induced a CTL response.

While the levels of CTLs generated by immunization with DNA and recombinant poxvirus appeared similar, it is difficult to compare the doses used because of the different nature of the two vectors. Nevertheless, whereas 100 $\mu$g of DNA and $10^7$ virus were sufficient to induce CTLs, in both cases, a 10-fold lower dose failed to induce CTLs; i.e. neither 10 $\mu$g of DNA [*Figure 3*(A)], nor $10^6$ vCP207 (data not shown) reproducibly induced detectable CTLs. It is also worth bearing in mind that DNA induced CTLs when given intradermally or intramuscularly, which are inherently more practical and less likely to raise safety concerns

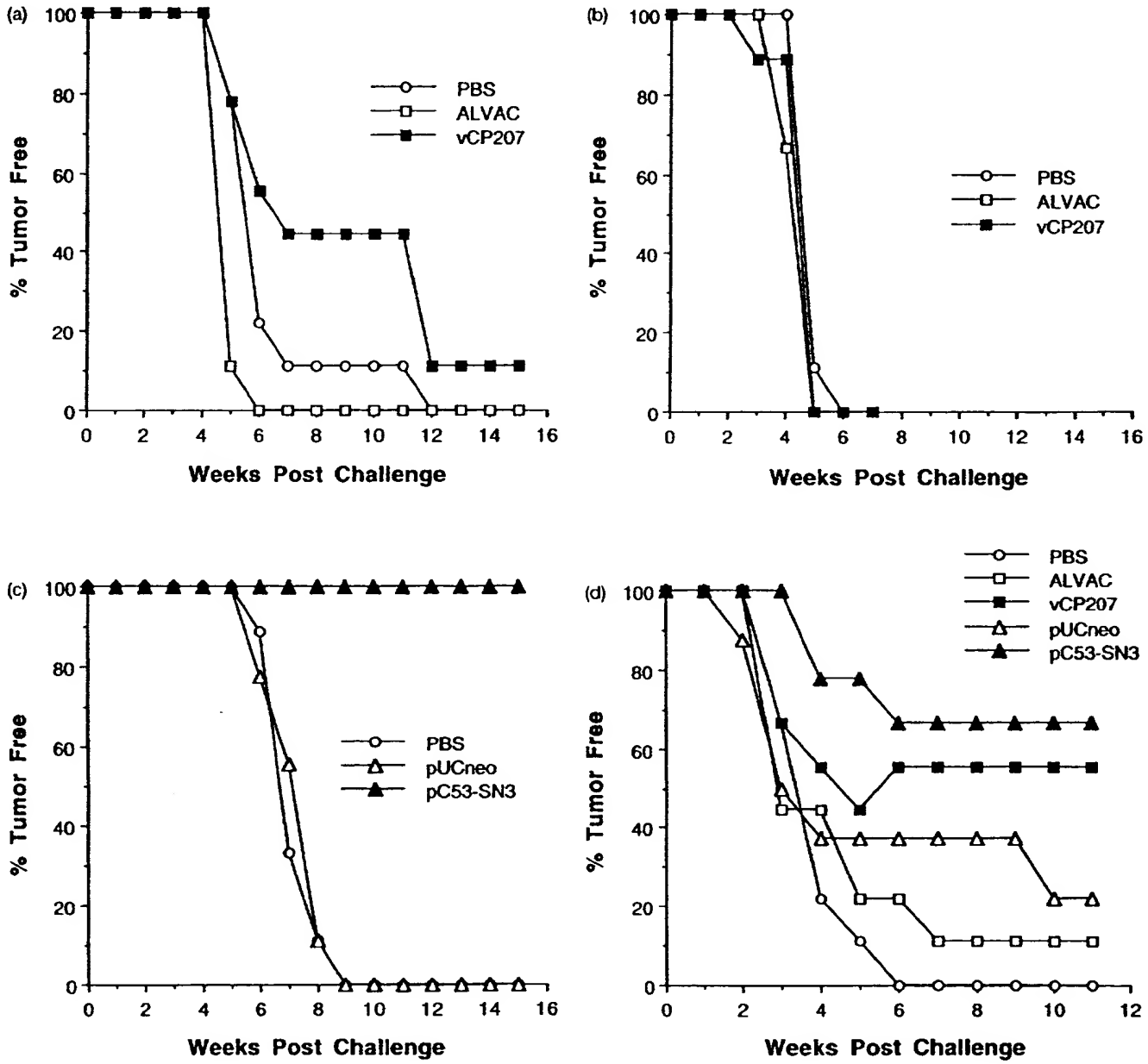


**Figure 4** Protection against challenge following immunization with vCP207 or pC53-SN$_3$. Mice (nine per group) were immunized at 0, 3 and 6 weeks with $10^7$ TCID$_{50}$ of vCP207 or ALVAC intravenously (A, B and D), or 100 $\mu$g of pC53-SN$_3$ or pUCneo intradermally (C and D) or with PBS. Mice were challenged subcutaneously 2 weeks after the last injection with $10^7$ cells of clone 2-21 (A and C), $10^7$ cells of untransfected BALB/3T12-3 (B) or $10^4$ cells of clone 4D5 (D). BALB/c mice were used in (A, B and C) and DBA/2 mice in (D)

than the intravenous route used with the recombinant virus. The good response to DNA may be related to the presence of immunostimulatory sequences (ISSs) consisting of unmethylated CpG dinucleotides within a PuPuCGPyPy sequence context. DNA or oligonucleotides containing these ISSs have been shown by a number of authors to induce immunomodulatory cytokines, NK activity and tumor regression[33-37]. In particular, the sequence 5'-AACGTT-3', which is present in pC53-$SN_3$ and pMP1406hup53 as well as in pUCneo, was noted as having a very strong effect on the immune response to intradermally administered DNA[33]. The immunostimulatory properties of DNA may explain the partial protection seen with vector DNA in the 4D5 challenge, since the parental P815 is sensitive to LAK killing, despite its high-level MHC-I expression[38].

Despite generating CTLs, intravenous immunization with vCP207 provided only partial protection in the transfected 3T3 and P815 models. These findings contrast with the results of Roth *et al.*, who reported protecting approximately 75% of the mice against a human p53-transfected BALB/c fibroblast line following only two subcutaneous immunizations with the same vCP207[7]. They did not report finding any CTLs, consistent with our seeing no CTLs, or protection (data not shown), after subcutaneous immunization. Although one cannot exclude the possibility that antibodies may be involved in protection, p53 is a nuclear protein, and thus we consider it much more likely that protection is mediated by CTLs. This is supported by the fact that, whereas only some mice immunized with p53 DNA made a detectable antibody response, all made CTLs and all were protected against challenge with the 2-21 line. Given the lack of a demonstrable CTL response in the experiments of Roth *et al.*, how might their observed protection be explained? Their challenge line, 10(3)273.1NT24, unlike our 2-21 and 4D5 lines, lacks endogenous murine p53 and expresses much higher levels of human p53 than ours (data not shown), which may render it more susceptible to weak anti-p53 responses. In this regard, it is interesting that Roth *et al.* saw down-regulation of p53 expression in the breakthrough tumors in immunized mice[7].

Since both recombinant ALVAC and DNA induce similar anti-p53 CTL responses, how might one explain the apparently superior protection induced by DNA in mice? Since the lysis assay is not quantitative, we cannot rule out the possibility that DNA may have induced a higher level of CTLs than the recombinant virus. Alternately, the CTLs may be qualitatively different, perhaps in their affinity for peptides presented on target cells, and this difference may be more important *in vivo* than *in vitro*. Of course, *in-vivo* protection may be mediated by other immune effectors in addition to CTLs. We do not believe that the ability of DNA to induce complete protection in the 2-21 model can be explained by an immune response to epitopes in the $neo^R$ gene shared by the immunizing plasmid and the transfected cell lines. First, mice immunized with the pUCneo vector developed tumors with kinetics identical to mice immunized with PBS, and their splenocytes did not lyse 2E4 at levels above background. Second, we induced a clear CTL response immunizing with pMP1406hup53, which lacks the $neo^R$ gene. It should be borne in mind, however, that vector/host interactions may be different in other species.

We have used human p53 in mice as a model system for cancer immunotherapy. However, whereas human and murine p53 are 81% homologous at the amino acid level[39], human p53 is not truly a self antigen in mice as it is in humans. The ability of mice to respond to wild-type epitopes of murine p53[26,27], the antibody and proliferative responses to wild-type p53 found in some cancer patients[14-17] and the fact that CTLs against wild-type epitopes can be elicited from donor PBLs[18-20], suggest that tolerance to this antigen can be broken. We have seen an antibody response in mice immunized intravenously with ALVAC encoding murine p53, although it is weaker than in mice immunized with the human recombinant. We are also examining the immune response to human p53 in monkeys, where the protein is >95% homologous to the endogenous monkey p53[39]. In the final analysis, however, whether one can elicit good immune responses to human p53 in cancer patients, who may, in some cases, already be primed by their tumors, and whether such responses might be of clinical benefit, can only be addressed in human clinical studies.

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# EXHIBIT H

antibody (Pharmingen clone) added to the wells. The wells were washed again and streptavidin–alkaline phosphatase was added. After further washing, spots were developed by adding an alkaline phosphatase substrate. The reaction was stopped by washing the wells and spots were counted under a stereomicroscope.

### 2.7. P. berghei challenge

Sporozoites of *P. berghei* (ANKA strain clone 1) were obtained from laboratory reared female *Anopheles stephensi* mosquitoes maintained at 18 °C for 20–25 days after feeding on infected mice. Salivary glands from the mosquitoes were collected by dissection and placed in a tissue homogeniser with RPMI 1640 (Sigma) to release the sporozoites, which were then counted using a haemocytometer. Mice were challenged by injection of 2000 sporozoites into the tail vein. Infection was determined by the presence of ring forms in Giemsa-stained blood smears taken 7 and 9 days post-challenge. If blood-stage parasitaemia was observed at two time points, the mice were sacrificed. Surviving animals were observed for, at least, a further 3 weeks for the development of malaria symptoms, to ensure that sterile immunity had been achieved rather than a delay in development of blood-stage parasitaemia. No mice that remained free of parasites on day 9 subsequently developed symptoms of infection or died.

## 3. Results

### 3.1. Immunogenicity of single adenovirus immunisations using different routes of administration

Initially the effect of the route of administration on the ability of Ad-PbCS to induce pb9-specific $CD8^+$ IFN-γ secreting T cells was tested. Rodrigues et al. [15] had found that high levels of malaria-specific $CD8^+$ T cells were induced after i.m. and s.c. immunisation, but not i.v., i.p. or intranasal (i.n.). We did not test i.v. or i.p. as these are not suitable routes for a prophylactic vaccine to be used in humans, but included i.d. and 'gene paint' groups. It has previously been demonstrated that simple administration of recombinant adenovirus onto the skin (known as gene painting) can induce an immune response against the antigen expressed by the virus [16]. Groups of mice received a single immunisation of $10^7$ PFU Ad-PbCS and splenocytes were tested for peptide-specific IFN-γ secreting T cells after 14 days.

The numbers of peptide-specific IFN-γ secreting T cells (Fig. 1) detected after i.m. or i.d. immunisation were somewhat higher than those detected after a single immunisation with i.m. DNA, and slightly lower than those detected after i.m. MVA [10]. Intranasal or s.c. immunisation produced very low numbers of peptide-specific IFN-γ secreting T cells, and none could be detected in the 'gene-paint' group. Intradermal immunisation was used for all subsequent experiments.

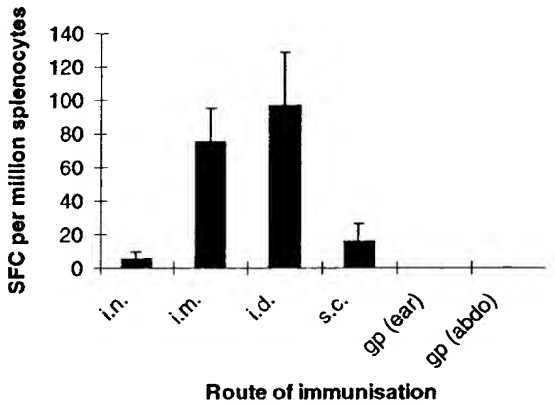


Fig. 1. Peptide-specific IFN-γ secreting T cells primed by a single immunisation of Ad-PbCS. Groups of three mice were immunised via the routes shown using $10^7$ PFU. The 'gp' refers to 'gene painting', i.e. painting the virus onto the surface of the skin of the ear or the abdomen. Elispot assays to detect IFN-γ secreting pb9-specific T cells were performed in duplicate on splenocytes after 2 weeks. Results are shown as SFC per million splenocytes.

### 3.2. Immunogenicity of different prime–boost immunisations

Fig. 2 shows the numbers of peptide-specific IFN-γ secreting T cells detected in the spleens of immunised mice receiving a priming immunisation on day 0 and a boosting immunisation on day 14. Using the same vaccine to prime and boost resulted in an increase in specific $CD8^+$ T cells, but a far greater increase was seen after heterologous boosting. As reported previously [5], DNA does not boost an existing response. However, the combination of adenovirus priming and MVA boosting resulted in extremely high numbers of peptide specific $CD8^+$ T cells. In addition to being able to prime a response that could be boosted to such high levels, Ad-PbCS was able to boost a response that had been primed by DNA or MVA.

### 3.3. Immunogenicity of triple combination immunisations

Heterologous priming and boosting is clearly much more effective than using the same vaccine repeatedly. It appeared possible that even higher numbers of $CD8^+$ cells could be stimulated by using three different vaccines sequentially. As DNA vaccines do not boost detectably, two triple immunisation regimes were tested; DNA/Ad/MVA and DNA/MVA/Ad. Groups of mice were immunised at 10 day intervals and splenocytes were tested 10 days after the final immunisation. As in the previous experiment high numbers of peptide-specific IFN-γ secreting T cells were detected after DNA/MVA, Ad/MVA, MVA/Ad and DNA/Ad immunisations (Fig. 3). However, these numbers

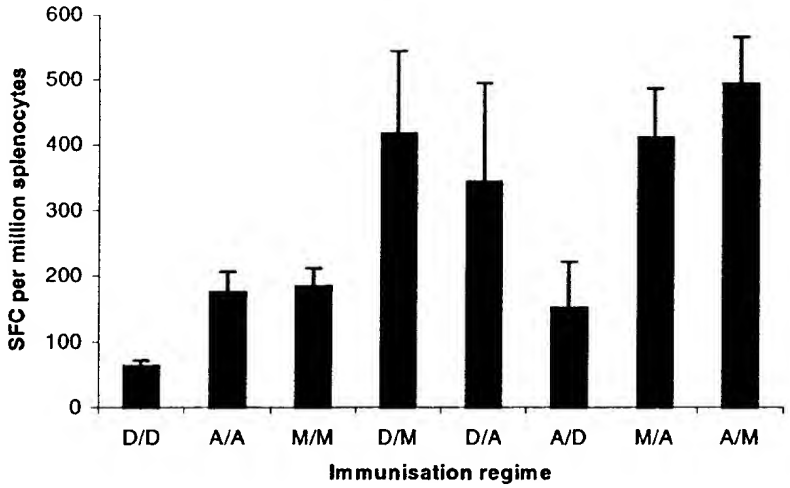


Fig. 2. Prime–boost immunisations. Groups of three mice were immunised on day 0 with the first vaccine shown (D: pSG2.PbCS; A: Ad-PbCS; M: MVA-PbCS), and day 14 with the second vaccine. DNA was injected i.m., adenovirus and MVA i.d. Elispots were performed on splenocytes isolated on day 28. Results are shown as SFC per million splenocytes.

were not increased above the immunogenicity observed with just two different vaccines when a third heterologous boosting immunisation was given.

### 3.4. Protection against infectious challenge

Several of the prime–boost combinations employing adenovirus as either a priming or boosting agent had resulted in high numbers of peptide-specific IFN-γ secreting T cells that would be expected to protect mice against challenge with *P. berghei* sporozoites. Therefore, groups of eight to eleven mice were immunised with a number of different heterologous prime–boost combinations and challenged with 2000 *P. berghei* sporozoites 2 weeks after the boosting immunisation. The results are shown in Table 1.

Intradermal administration of adenovirus followed by i.d. MVA completely protected the immunised mice. In earlier *P. berghei* challenge experiments we had found that i.m. DNA followed by i.d. MVA gave a high level of protection, but that

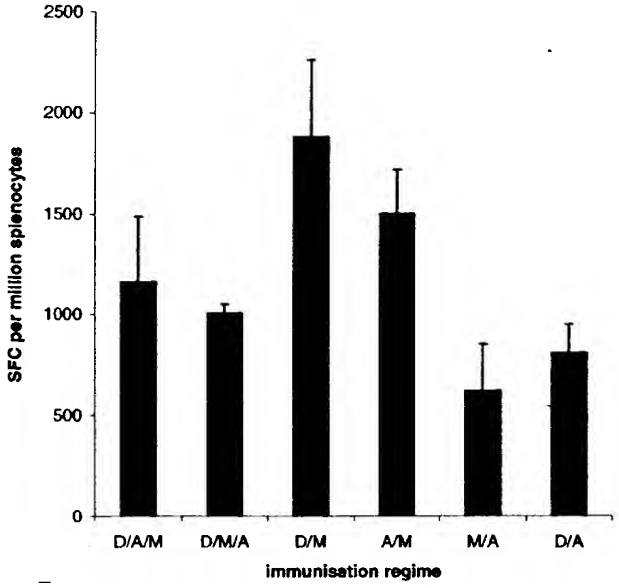


Fig. 3. Triple combination immunisations. Groups of three mice were immunised at 10 day intervals with the vaccines shown, with the first vaccine of the double combinations given on the same day as the second vaccine of the triple combinations. Elispot assays were performed in duplicate on splenocytes 10 days after the last immunisation.

Table 1
Protection of mice immunised with different heterologous prime–boost combinations[a]

| Prime | Boost | No. infected | No. challenged | Protection (%) |
|---|---|---|---|---|
| DNA | MVA | 5 | 10 | 50 |
| Ad | MVA | 0 | 10 | 100 |
| MVA | Ad | 2 | 10 | 80 |
| Ad | Ad | 7 | 8 | 13 |
| DNA | Ad | 5 | 11 | 55 |
| MVA | MVA | 5 | 8 | 38 |
| Ty (i.v.) | Ad | 7 | 10 | 30 |
| Ty (i.v.) | MVA (i.v.) | 1 | 11 | 91 |
| Ty | Ad | 4 | 10 | 60 |
| Naïve | | 8 | 10 | 20 |

[a] DNA was given intramuscularly (50 μg dose), MVA ($10^6$ ffu dose) adenovirus ($10^7$ PFU dose) and Ty VLPs (100 μg dose) were administered intradermally unless otherwise stated. The priming immunisation was given on day 0, the boosting immunisation on day 14, and the challenge carried out on day 28 with 2000 *P. berghei* sporozoites injected into the tail vein. Mice were scored for presence or absence of blood stage parasitaemia by blood films taken on days 7 and 9 post-challenge. Mice that did not have blood stage parasites by day 9 did not subsequently develop blood-stage malaria.

complete protection was only achieved when the MVA was administered intravenously [5]. However, using adenovirus priming and MVA boosting, both vaccines can be given intradermally with no loss of protection. MVA priming and adenovirus boosting also resulted in a high level of protection, whereas two sequential adenovirus immunisations did not. Adenovirus also boosted responses primed by DNA or Ty VLPs resulting in levels of protection comparable to that obtained by DNA priming and i.d. MVA boosting.

## 4. Discussion

Much research effort has been directed towards identifying pre-erythrocytic antigens of *P. falciparum* [17–21], finding $CD8^+$ epitopes preferably in conserved regions of these antigens [22–24] and testing naturally exposed individuals for $CD8^+$ responses to those epitopes [25–29], all with the aim of developing a vaccine that would be effective against the pre-erythrocytic stages of *P. falciparum* infection. Such a vaccine would control infection before any symptoms of the disease became apparent and would be suitable for people living in malaria-endemic areas as well as travellers to those areas. However, in order to utilise the results of this work, it is necessary to identify a means of vaccination capable of inducing a strong $CD8^+$ T cell response using materials suitable for a human prophylactic vaccine.

In an early comparison of delivery systems all utilising the CS protein from *P. berghei* [30] Ty VLPs and lipopeptides [31] were found to induce $CD8^+$ T cell responses reliably. In subsequent challenge experiments immunisation with Ty VLPs failed to provide protection [6]. Lipopeptides are not well-suited for use as a malaria vaccine as individual lipopeptides would need to be prepared for each epitope specific for a single HLA type, and a large number of these would be required to protect a genetically diverse human population. In addition, large scale manufacturing of lipopeptides is expensive and technically difficult. Partial protection from infection has been observed using DNA vaccines in the *P. yoelii* model [3,32] but very little or no protection is observed using DNA vaccines in the *P. berghei* model [5]. Recombinant MVA expressing *P. berghei* CS was more immunogenic but failed to protect against infection [5].

Complete protection was achieved by using one vaccine (DNA or Ty VLPs) to prime, and another (MVA) to boost. This resulted in 100% (i.m. DNA/i.v. MVA [5] or i.v. Ty VLP/i.v. MVA [33]) protection against infection in the *P. berghei* model, using materials suitable for immunising humans. This protection was associated with a striking enhancement of induced specific $CD8^+$ T cells in splenocytes. The reverse order of immunisation (MVA first followed by Ty VLP or DNA second) was markedly less protective and less immunogenic. The mechanism of this effect is uncertain but, we have proposed that an immunodominance effect is relevant. In this scenario, a "focusing" of the immune response on an epitope in the foreign antigen by an initial DNA immunisation allows the more immunogenic MVA recombinant vaccine to enhance this pre-primed CD8 T cell response in preference to CD8 T cell responses to epitopes in vaccinia antigens.

However, these very high levels of protection in heterologous prime–boost immunisation regimes were obtained by using intravenous immunisation with MVA and Ty VLPs. This route may be suitable for a therapeutic cancer vaccine which is administered to a small number of patients receiving hospital treatment, but would not be suitable for a prophylactic malaria vaccine intended for use by large numbers of people. Intradermal immunisation with MVA was also tested, but resulted in somewhat lower levels of protection.

We now report 100% protection using two recombinant, replication-deficient viruses, both administered intradermally with recombinant replication-deficient adenovirus used for the priming immunisation. Replication competent adenovirus has been successfully and safely used to immunise large numbers of people [34–36] but the use of such a vaccine could potentially cause an adverse reaction in immunocompromised individuals. However, by using a replication-deficient virus, there is probably no possibility of a disseminated infection occurring. The antigen delivery system used for the boosting immunisation, MVA, is also a very safe vaccine that was used during the smallpox eradication campaign [7].

Intradermal administration of adenovirus and MVA vaccines may be achieved by using a needle to inject a suspension of the virus. Another possibility is the use of a needle-less injection device to administer a freeze-dried powder containing the vaccine. Preliminary work with MVA has shown this to be feasible (P. Dégano, personal communication) and this raises the possibility of manufacturing individually prepared doses of the vaccine that do not need cold storage. This would be a great advantage for a vaccine that is needed in rural areas of Africa.

The lack of boosting after repeated immunisations with the same recombinant virus is probably in part related to the anti-viral antibody and cellular immune response that is primed by the first immunisation. In subsequent immunisations, these antibodies may prevent the virus from entering cells and if the boosting dose of the virus succeeds in infecting host cells, the pre-existing response to all the viral antigens will be boosted, rather than only the response to the recombinant antigen. However, priming with one virus and boosting with another allows the second virus to infect cells, express the recombinant antigen and boost the response to the only antigen common to both viruses, resulting in a very high anti-malaria $CD8^+$ T cell response. Many antigen delivery systems can prime a response, but we have only been able to achieve significant boosting of a $CD8^+$ T cell response using a recombinant poxvirus or adenovirus. Antigen delivery systems such as DNA and Ty VLPs which do not express any genes in the host cell other than the *Plasmodium* antigen fail to create optimal conditions for boosting. Study of cytokines and chemokines expressed at the site

of boosting may lead to elucidation of the mechanism of boosting.

Intriguingly, a second heterologous boosting using either recombinant MVA or adenovirus did not boost the already high numbers of pb9-specific $CD8^+$ T cells present after heterologous prime–boost. One possible explanation is that an upper limit of $CD8^+$ T cells had already been reached by the prime–boost and that some autoregulatory mechanism prevents further enhancement of this level. Alternatively or additionally, a qualitative change in the T cells induced by the first boost may render them refractory to further amplification. It may be possible to address this possibility through adoptive transfer studies. It is possible that triple heterologous immunisations would be more effective than double when employing less immunogenic antigens.

Enhanced T cell responses have been reported following priming with recombinant influenza and boosting with recombinant replication competent vaccinia viruses [37]. However, the reverse order of immunisation was not protective and the recombinant influenza virus was unable to boost CD8 T cells substantially [38]. Increased humoral responses have been achieved using DNA to prime and recombinant ovine adenovirus to boost [39] or recombinant vaccinia to prime and adjuvanted protein to boost [40]. However, these regimes rely on the use of replication competent viruses which may not be safe to use in humans. Using DNA to prime and recombinant fowlpox to boost [41] has been shown to increase humoral responses. Boosting of humoral responses at mucosal surfaces using recombinant replication-defective adenovirus administered intranasally has also been demonstrated [42]. The generation of a strong $CD8^+$ T cell response using DNA priming followed by MVA boosting has now been demonstrated with epitopes from several infectious organisms, including HIV [43], influenza and mouse tumour epitopes [44], and *Mycobacterium tuberculosis* [45]. The high immunogenicity of both the recombinant adenovirus-recombinant MVA immunisation regime and the opposite order of immunisation now questions the importance of the simple immunodominance model of the mechanism of heterologous prime–boost immunisation outlined above. Both MVA and adenoviral vectors will probably prime responses to several viral as well as the malarial epitope and yet in each case the other virus, unlike recombinant Ty VLPs or plasmid DNA, can boost the malaria-specific CD8 T cell response substantially. It appears that some properties intrinsic to adenovirus and poxvirus vectors but missing from the other delivery systems are required for substantial boosting of primed $CD8^+$ T cells.

Adenovirus and MVA are both viruses with an excellent safety record in human immunisations. The generation of recombinant viruses can be accomplished simply, and they can be manufactured reproducibly in large quantities. A potential disadvantage of adenovirus vectors is the existence of naturally generated immunity to many serotypes of adenoviruses in older children and adults. However, in young children, the main target population for a vaccine aimed at reducing global malaria mortality, this may be much less of a problem particularly if a recombinant adenovirus were administered soon after waning of maternally transmitted antibodies. Intradermal administration of recombinant replication-deficient adenovirus followed by recombinant MVA therefore could be suitable for prophylactic vaccination of humans against malaria and possibly other diseases which can be controlled by a $CD8^+$ T cell response.

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# Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime–boost immunisation regimes

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**Abstract**

Recombinant replication-defective adenovirus expressing the CS gene from *Plasmodium berghei* (Ad-PbCS) was found to induce a strong $CD8^+$ T cell response after intra-dermal or -muscular immunisation. Boosting of an adenovirus-primed immune response with the replication-impaired poxvirus, modified vaccinia virus Ankara (MVA) led to enhanced immunogenicity and substantial protective efficacy. The recombinant adenoviral vaccine was capable of boosting to protective levels a $CD8^+$ T cell response primed by either a plasmid DNA vaccine, a recombinant Ty virus-like particle vaccine or recombinant MVA each expressing the same epitope or antigen. Complete protective efficacy after intradermal immunisation was observed with the adenovirus prime–MVA boost regime. This study identifies recombinant replication-defective adenovirus as an alternative to recombinant replication-defective poxviruses as boosting agents for the induction of strong protective $CD8^+$ T cell responses. 

*Keywords:* Immunogenicity; Adenoviral vector; Heterologous prime–boost

## 1. Introduction

Studies on the pre-erythrocytic liver stage of *Plasmodium* infections have implicated $CD8^+$ T cells in protection against the infection. In general, live attenuated vaccines capable of producing a short-lived infection that is harmless in a healthy individual are effective at inducing T cell responses. Irradiated *Plasmodium* sporozoites, which can infect hepatocytes but do not progress to a blood-stage infection have been shown to protect both mice and humans against malaria by inducing T cell responses against pre-erythrocytic antigen [1]. However, in humans this requires multiple immunisations over a long period of time and, whilst these experimental immunisations have provided valuable information, this approach cannot be used to produce a vaccine to be deployed widely for prophylactic immunisation.

Recombinant protein sub-unit vaccines with adjuvant generally elicit humoral responses, but poor $CD8^+$ T cell responses [2]. DNA vaccines have been shown to elicit both humoral and cellular responses in mice against the *P. yoelii* CS protein [3]. However, mice immunised with a DNA vaccine expressing the *P. berghei* CS gene had only a weak $CD8^+$ T cell response to the protective $CD8^+$ epitope pb9 [4] and were not protected against challenge with infectious sporozoites even after repeated immunisations [5]. Ty virus-like particles (Ty VLPs), consisting of a recombinant protein assembled into a 30 nm particle, induce stronger $CD8^+$ T cell responses, but do not protect against infection [6]. Recombinant viruses can also be used as vaccines. Modified vaccinia virus Ankara (MVA) does not replicate in human cells and is a very safe virus to use as a vaccine [7–9] Recombinant MVA expressing *P. berghei* CS has also been tested in mice, resulting in similar levels of peptide-specific lysis in a cytotoxicity assay to mice immunised with Ty VLPs [10]. Again, these mice were not protected against challenge with 2000 sporozoites administered by i.v. injection. However, despite the fact that neither DNA vaccines, Ty VLPs or MVA used alone can protect against *Plasmodium* infection, using either DNA

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or Ty VLPs to prime a T cell response and MVA to boost it results in greatly increased numbers of IFN-γ secreting $CD8^+$ T cells, and complete protection against infection when the MVA is administered intravenously [11].

Replication-deficient adenovirus derived from human serotype 5 has been developed as a live viral vector by Graham and colleagues [11,12]. Adenoviruses are non-enveloped viruses containing a linear double stranded DNA genome of around 36 kb. Recombinant viruses can be constructed by in vitro recombination between an adenovirus genome plasmid and a shuttle vector containing the gene of interest together with a strong eukaryotic promoter in a permissive cell line which allows viral replication. High viral titres can be obtained from the permissive cell line, but the resulting E1-deleted viruses, although capable of infecting a wide range of cell types, do not replicate in any cells other than the permissive line, and are therefore safe antigen delivery systems. Recombinant adenoviruses have been shown to elicit protective immune responses against a number of antigens including tick-borne encephalitis virus NS1 protein [13] and measles virus nucleoprotein [14]. Further, a single dose of recombinant adenovirus resulted in an impressive 93% decrease in the level of hepatic parasite rRNA of *P. yoelii* in mice, and 40% protection, which was shown to be $CD8^+$ T cell-mediated [15].

We have constructed a recombinant replication-deficient adenovirus expressing the CS gene of *P. berghei* (Ad-PbCS) and tested the capacity of this virus to induce $CD8^+$ T cell responses in mice either alone or in combination with other types of vaccines. As expected, when used as a single immunisation high levels of antigen-specific $CD8^+$ T cells were generated. Adenovirus priming followed by MVA boosting resulted in complete protection. However, surprisingly, adenovirus was able to boost a response primed by DNA, Ty VLPs or MVA, thus, demonstrating that recombinant adenoviruses can be used as alternatives to poxviruses for boosting $CD8^+$ T cell responses.

## 2. Material and methods

### 2.1. DNA vaccine

The DNA vaccine pSG2.PbCS consists of the CMV promoter with intron A driving expression of *P. berghei* CS protein, with the bovine growth hormone poly A sequence. The plasmid is kanamycin resistant and incapable of replication in eukaryotic cells. Plasmids were prepared using Qiagen columns and diluted in endotoxin-free phosphate-buffered saline (PBS).

### 2.2. Construction of recombinant replication-deficient adenovirus

The CMV promoter with intron A, *P. berghei* CS protein gene and bovine growth hormone poly A sequence from pSG2.PbCSP was ligated into the multiple cloning site of the adenovirus shuttle vector pDE1sp1A [12]. This vector can be used to construct Adenovirus 5 recombinants with deletions in E1. The recombinant shuttle vector was used to transfect the permissive cell line 293 along with the adenovirus genome plasmid pJM17 [11]. Virus from transfected cells was clonally purified by three successive limiting dilution in 293 cells, and expression of *P. berghei* CS in cells infected with the isolated virus was confirmed by immunofluoresence. Large quantities of virus were prepared from infected 293 cells and purified by extraction with Arklone [11] prior to immunisation.

### 2.3. Ty VLPs

Recombinant Ty VLPs expressing the pb9 epitope from *P. berghei* CS, SYIPSAEKI, were prepared as described in [6] and suspended in PBS.

### 2.4. Recombinant MVA

MVA expressing *P. berghei* CS was prepared by in vitro recombination between a shuttle vector containing the CS gene driven by the vaccinia P7.5 promoter and MVA virus in primary chick embryo fibroblasts [9]. The recombinant, which also expresses *Escherichia coli* β-galactosidase, was repeatedly plaque purified and expression of the recombinant gene was confirmed by immunofluoresence. Virus for immunisation was purified by ultracentrifugation through a sucrose cushion and suspended in endotoxin-free PBS.

### 2.5. Immunisations

Female BALB/c mice 4–6-week-old were immunised under anaesthesia as described for individual experiments. Intramuscular DNA immunisations used 50 μg DNA in each musculus tibialis. MVA and adenovirus ($10^6$ and $10^7$ PFU per dose, respectively) were injected intradermally into the ear. Ty VLPs (100 μg per dose) were injected intradermally in the foot-pad or intravenously into the lateral tail vein.

### 2.6. ELISPOT assays

The number of IFN-γ secreting, pb9-specific T cells in fresh splenocyte preparations was determined as described previously [5] by coating 96-well nitrocellulose plates with anti-mouse IFN-γ antibody (clone R4 from ETCC), washing with PBS and subsequent blocking with complete medium containing 10% FCS (foetal calf serum). Splenocytes from immunised mice were resuspended at $1 \times 10^7$ to $2 \times 10^7$ cells/ml and placed in duplicates into the coated wells, and serially diluted. The H2-$K^d$-restricted peptide pb9 (SYIPSAEKI)[4] was added to test wells and an irrelevant peptide to control wells. After overnight incubation the wells were washed and a second, biotinylated anti-IFN-γ

# EXHIBIT I

# The multistep nature of cancer

BERT VOGELSTEIN AND KENNETH W. KINZLER

***Cancer is a distinct type of genetic disease in which not one, but several, mutations are required. Each mutation drives a wave of cellular multiplication associated with gradual increases in tumor size, disorganization and malignancy. Three to six such mutations appear to be required to complete this process.***

One of the most important developments in genetics over the past decade has been the proof that cancer is, in essence, a genetic disease. However, there are two key differences between cancer and most other genetic diseases. First, cancer is, for the most part, caused by somatic mutations, whereas all other genetic diseases of mammals (excluding those involving mitochondrial genes) are caused solely by germ-line mutations. Second, each individual cancer arises not from a single mutation, but from the accumulation of several mutations. This 'multi-hit' concept is central to understanding neoplasia, but only in the past decade has it become possible to provide support for this concept at the molecular level. Here, we review some of the many studies that have substantiated this view.

## Epidemiology

If one plots the incidence of most common human cancers against age, a striking relationship is observed: the incidence rate increases dramatically ($10^3$–$10^7$ times) with age (Fig. 1). Although there are many possible explanations for the exponential relationship, the most attractive is that three to seven 'hits' are required for a cancer to form[1]. These 'hits' could represent insults to separate cells, but because each cancer appears to arise from a single cellular progenitor (clonal growth) it is more likely that they represent sequential mutations of growth-regulatory genes in a single cell and its progeny.

According to this idea, tumors grow by a process of clonal evolution driven by mutation[2]. The first mutation would result in limited expansion of the progeny of a single cell. One of these cells would later acquire a second mutation, perhaps allowing growth of a small benign tumor. One cell within this benign tumor would then undergo a third mutation, overgrow its sister cells, and form a more advanced tumor composed of progeny cells with three mutations. Eventually the cell will accumulate a sufficient number of hits to make it malignant, enabling it to invade surrounding tissues and metastasize to other organs (the latter properties alone distinguish malignant tumors, cancers, from benign tumors). In this sequential multi-hit model of carcinogenesis, the fact that most cancers occur in older people is explained by the decades required for an individual to accumulate the number of mutations necessary to cause malignancy.

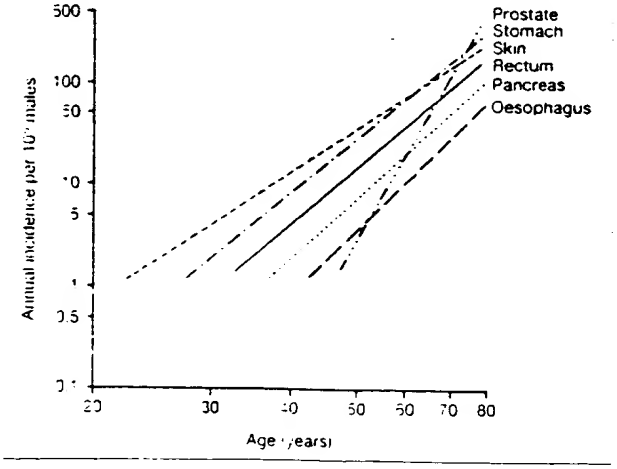


*FIG* 1

Cancer incidence versus age. The log of the incidence rate and the log of age have a linear relationship, with the incidence increasing according to a power of age. Modified from Ref. 1.

Other epidemiological and clinical observations were critical in formulating the multi-hit hypothesis. For example, patients exposed to radiation often develop cancer, but the cancers do not form immediately. In the case of patients who underwent X-ray therapy for tuberculosis, breast cancers develop an average of 15 years after the initial exposure[3]. Why the long time lag? One explanation is that the radiation induced a mutation in a cell, but additional mutations in the progeny of this cell were required for a cancer to form. Again, because of the low incidence of additional mutations after radiation therapy had ended, long periods were required for the cancer to appear.

Morphological observations are also in accord with the multi-hit hypothesis. In the colon, the gradual evolution of tumors is well documented. Small benign tumors (adenomas) are the first manifestations of neoplasia in colorectal epithelium. These tumors are only a few millimeters in diameter and are almost normal in their intra- and intercellular organization. With time, these tumors grow and their cells become more disorganized. Eventually, the tumor evolves into a cancer (carcinoma), presumably because one of the cells in the adenoma has acquired a sufficient number of mutations to drive the processes of invasion and metastasis. These morphological observations on colonic tumors have now been supplemented by documentation of the mutations that are associated with initiation and progression[4]. For example, it has been shown that malignant cells within a single tumor have the same set of mutations found in benign portions of the tumor, but with the addition of at least one further mutation that is absent in the benign precursor cells. A similar evolution of morphology has been observed in cervical carcinomas. The cervical smear test is used to detect neoplastic cells shed from the cervix at a stage before full-blown malignancy. Surgery at this stage can be life-saving, whereas once the tumor has acquired the ability to metastasize through sequential mutation, the resultant cancers are often incurable.

The examples given above suggest that multiple mutations occurring over decades drive the neoplastic process. Exceptions to this general scenario provide

important lessons. Tumors that occur in children obviously do not take decades to develop. The timing of tumorigenesis in such cases stimulated Knudson to propose his now well-accepted model for neoplastic development[5]. The model invokes two important principles. First, in childhood tumors of the eye and kidney, only two mutations are rate limiting for cancer formation. Second, either the two mutations can both develop somatically, or one can be inherited and the other somatic. In the latter case, every eye or kidney cell of the individual has a 'head start' on the neoplastic process, and such individuals have a high risk of developing these specific cancers. One important finding from these pediatric studies is that the number of hits required is likely to vary in different cell types. Similarly, it may vary in different species. For example, rodent cells are generally easier to transform than human cells, and this may be because fewer hits are required.

## Transformation *in vitro*

The morphological and epidemiological observations noted above only indirectly support the multihit scenario. More direct evidence is provided by gene transfer experiments. An oncogene can be operationally defined as a gene whose activity leads to enhanced cell growth. If an oncogene is transferred to cultured primary rodent fibroblasts under standard conditions, no changes in growth are observed. However, if two oncogenes are transferred simultaneously, the recipient cells grow abnormally well *in vitro*, forming foci of piled-up cells. These foci are tumorigenic when implanted into mice[6]. Moreover, not all combinations of oncogenes will transform cells, so that oncogenes can be classified by their ability to complement one another in transformation assays. This suggests that the cell has evolved several growth control circuits, and more than one circuit must be damaged before abnormal growth ensues. Thus, an oncogene that disrupts one circuit can be complemented in transformation assays by a gene that disrupts a second circuit, but if both oncogenes act through the same circuit the cell maintains sufficient control to prevent neoplastic transformation.

An interesting new example of this synergy involves c-*myc* and *bcl-2*. Overexpression of c-*myc* is often associated with neoplastic growth. However, c-*myc* overexpression also has another effect: while cells grown under limiting conditions, such as serum starvation, are normally blocked at G0 or G1 in the cell cycle, cells that overexpress c-*myc* undergo apoptosis under these conditions. Overexpression of *bcl-2* can rescue these cells from premature death[7,8]. Tumor cells *in vivo* must frequently be in situations where growth factors are at low concentration, for example, in poorly vascularized areas or near necrotic portions of tumors. This may explain why alterations at the *bcl-2* locus often accompany c-*myc* overexpression in lymphomas. It is likely that other combinations of oncogenes act in a similar way to alter the complex balance between cell division and cell death that determines whether a subpopulation of tumor cells will expand.

One exception to the requirement for two separate oncogenes to transform primary cells is informative.

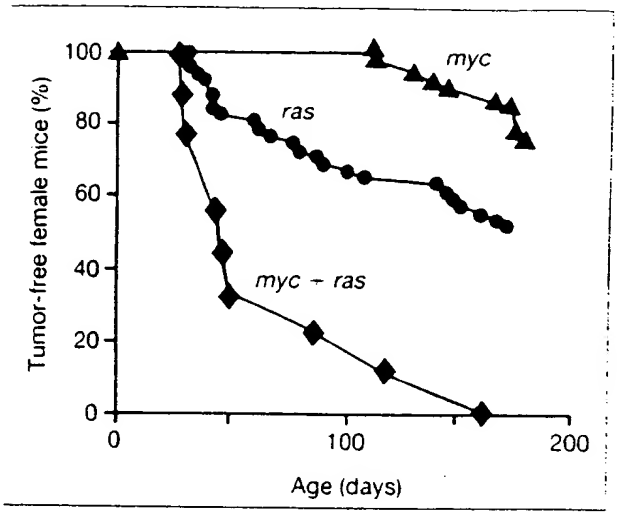


*FIG* 2

Oncogene synergism *in vivo*. Kinetics of breast tumor occurrence in mice transgenic for the *ras* or *myc* oncogene, and in mice doubly transgenic for *ras* and *myc*. Expression of *ras* and *myc* was under the control of the mouse mammary tumor virus breast-specific promoter. Values for $t_{50}$, the age by which 50% of mice had developed tumors, are as follows: *myc* transgenics, 325d (n = 50); *ras* transgenics, 168d (n = 52); *myc* + *ras* transgenics, 46d (n = 9). Reproduced, with permission, from Ref. 13.

When oncogenes are transfected together with genes encoding drug resistance, only cells that have incorporated the resistance markers can form colonies in the presence of drugs. Under these conditions, a single oncogene is sufficient for neoplastic transformation of the selected colonies[9]. It seems that in normal cells, cell–cell contact inhibits the growth of cells expressing a single oncogene, but if this cell contact inhibition is removed (by killing normal cells with a drug) one constraint on abnormal growth is removed and full transformation ensues[10]. Thus, the cellular environment (as well as the cell type and species) can modulate the number of hits required for tumorigenicity. One would expect, from these observations, that mutations that disrupt cell interactions might play an important role in promoting the growth of some cancers. This expectation has been realized: some mutations seen in human cancers reduce the expression of cell adhesion molecules, and artificial modulation of cell adhesion can promote tumor cell growth and/or invasion[11].

## Tumors in transgenic animals

Studies using transgenic mice have made an important contribution to our understanding of cancer over the past decade. When an oncogene such as *myc* is transferred to the mouse germ line under the control of a breast-cell specific promoter, the transgenic animals develop breast tumors[12]. However, of the thousands of breast tumor stem cells in the mouse, only one or two become neoplastic. This suggests that the presence of a single oncogene is not sufficient for tumorigenesis, even when the gene is expressed at constitutively high levels for long periods. However, doubly transgenic mice, made by breeding *myc* transgenics with mice

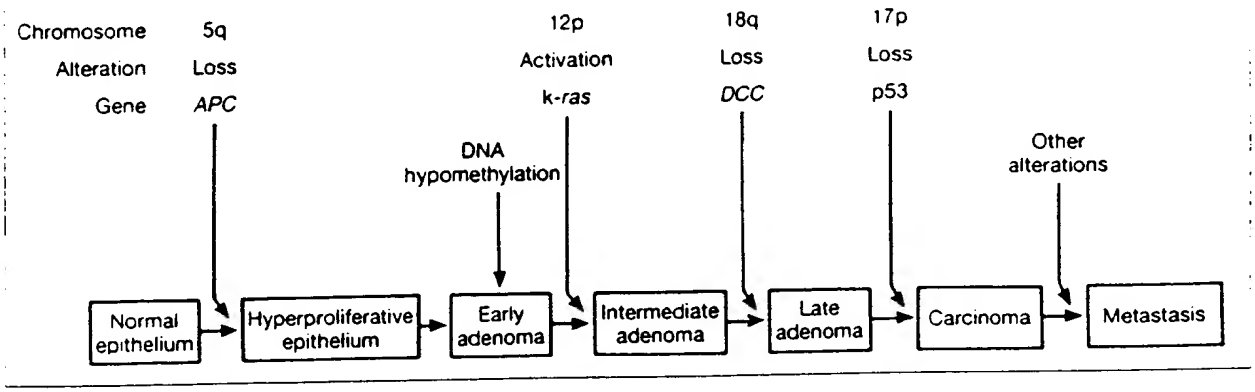


*FIG* 3

A genetic model for colorectal tumorigenesis. Modified from Ref. 4.

transgenic for a second breast-specific oncogene, develop tumors much earlier and more frequently (Fig. 2). Again, two oncogenes are more efficient than one. Even in the doubly transgenic mice, however, the proportion of breast cells that become neoplastic is still small, suggesting that further mutations are required to convert a normal breast epithelial cell into a cancerous one[13]. It will be of interest to determine how many transgenic oncogenes are required to convert all breast epithelial cells to malignancy, independent of additional somatic mutation.

## Tumors in humans

Studies of human tumors have added a new dimension to this story. In animals, the tumorigenic process has been analysed primarily in tumors deliberately induced by mutagenic agents or viruses. In humans, dissection of the process has proceeded through the characterization of mutations occurring in sporadic tumors, often involving genes identified by positional cloning approaches. Part of the revolution in cancer research in the 1980s was the discovery that the two approaches converge: the same kinds of genes are mutated in all tumors.

Much of the research on human cancers has centered on tumor suppressor genes. These genes are negative regulators of cellular proliferation and their inactivation by mutation results in the loss of a crucial 'brake' on tumor growth. To date, six presumptive suppressor genes, each mutated in a different spectrum of human cancers, have been identified: the retinoblastoma gene, *RB* (chromosome 13q); the Wilms' tumor gene, *WT1* (11p); the gene deleted in colon carcinoma, *DCC* (18q); the neurofibromatosis type 1 gene, *NF1* (17q); the p53 gene (17p); and the gene involved in familial adenomatous polyposis coli, *APC* (5q). Several other suppressor genes have been localized to specific chromosomal regions by linkage analysis in kindreds with inherited tumor predisposition syndromes or by the pattern of somatic chromosomal losses in tumors[14]. Such genes can be mutationally inactivated in the germ line, resulting in a predisposition to tumors, or, more commonly, undergo somatic mutation, leading to initiation or progression of sporadic tumors.

A good illustration of how the studies of experimental systems and human tumors have converged and an example of particular relevance to the multi-hit phenomenon, is provided by human papilloma viruses (HPVs). These viruses have at least two oncogenes (*E6* and *E7*) that can transform appropriate recipient cells in culture. Infection of human cervical cells with these viruses *in vivo* appears to be the first step in the pathway towards cervical cancer[15]. Infection may actually represent a double hit, because the E6 and E7 proteins bind to, and presumably inactivate, the products of two suppressor genes (the p53 and RB proteins, respectively). Only those HPV subtypes whose E6 and E7 proteins bind RB and p53 are associated with cancers in humans[16,17]. In cervical tumors that are not associated with HPV infection, p53 (and perhaps RB) may be inactivated by mutations, such as deletions, splice-site changes and codon substitutions, rather than by binding to a viral protein[18]. Progression of HPV-initiated cells to the fully malignant state requires additional hits in genes not yet identified.

Another example of the interaction between experimental and human tumor research is the characterization of chronic myelogenous leukemia (CML). One of the viruses that causes leukemia in mice carries a transduced and mutated version of a cellular gene, c-*abl*. The mutation results in constitutive activity of the abl tyrosine kinase. In CML in humans, the *ABL* gene on chromosome 9 is translocated to a locus on chromosome 22 (Ref. 19). This translocation results in the activation of the ABL tyrosine kinase, and is an early (but perhaps not the first[20]) genetic event in the development of CML. Additional genetic alterations, largely undefined at the molecular level, are required to convert the relatively indolent CML to the more aggressive forms that kill the patient; this is yet another example of the need for multiple hits in cancer evolution.

Numerous other common human tumors, including those of the breast, brain, lung, bladder, bone and colon, have been shown to have mutations at more than one gene. Because colon tumors evolve through well-defined morphological stages, it has been possible to establish the order in which mutations occur in this tumor type (Fig. 3). The development of colorectal tumors appears to be initiated by mutations at the *APC* tumor suppressor gene. These mutations usually cause truncation of the protein. It is not yet clear whether the second hit is a mutation at the remaining *APC* allele,

resulting in the total absence of functional gene product, or a mutation of some other, as yet unidentified, gene. Mutation of *APC* can occur somatically, causing initiation of a single colorectal tumor[21], or in the germ line, resulting in a predisposition to such tumors[22,23]. Patients who have a germ-line mutation of *APC* develop thousands of tumors throughout their colon: this is a remarkable demonstration of what can happen when a brake controlling cell growth is lost. Mutation at *APC* leads to the formation of benign adenomas which gradually grow bigger. Mutation of the *RAS* gene often occurs in one of these benign tumor cells, leading to a further clonal expansion. Sequential mutations in the *DCC* and p53 tumor suppressor genes appear to complete the process, driving waves of clonal expansion that finally result in progression from the benign to the malignant state. No stage of tumorigenesis is static, including the malignant (carcinomatous) stage: additional mutations occur, giving rise to small subpopulations that may not overgrow the entire tumor. These clonal subsets are one of the most difficult challenges in clinical oncology, as they are a reservoir of genetically heterogeneous cells with varying capacities for growth, differentiation and metastasis, and differential sensitivities to drugs, radiation and immune attack.

In all of the human tumors discussed above, mutations in one particular gene appear to precede those in others. Because the oncogenes and tumor suppressor genes so far identified appear to control different cell growth circuits, one might expect that the order in which the circuits are interrupted would be unimportant, and that so long as a sufficient number of critical pathways were disabled, tumor growth would ensue. In fact, although there is some variability in the order of mutations, there is also a clear preference. In CML, colorectal tumors and bladder neoplasias, respectively, mutations at *ABL* (Ref. 19), *APC* (Ref. 21), and a gene on chromosome 9q (Ref. 24) clearly precede other mutations. This suggests that only a subset of genetic pathways can initiate the tumorigenic process in particular cell types and that mutation at some genes confers a selective growth advantage only at later stages in tumor development. Moreover, in most epithelial cells, the neoplastic pathways appear to be guarded by suppressor genes, rather than oncogenes. The reverse may be true in hematopoietic cells, where mutation of an oncogene often appears to be the initiating event in neoplasia. Mutations at *RAS*, although powerful in some situations, appear to have little effect on the neoplastic transformation of epithelial cells in the absence of other critical changes. This explains why mutations of *RAS* in human tumors always occur as a late event that promotes tumor progression, rather than an early event that initiates the process.

## Prospects

One of the beauties of genetics as a science is the clear cause and effect relationships that can be inferred, relating genotype to phenotype. The genetics of cancer forces us to re-examine our simple notions of causality, such as those embodied in Koch's postulates: how does one come to grips with words like 'necessary' and 'sufficient' when more than one mutation [illegible] that phenotype can be produced by different mutant genes in various combinations? Similar problems arise in the analysis of other complex human diseases, such as those involving autoimmunity, atherosclerosis, hypertension and psychiatric disorders. Perhaps the conceptual lessons derived from the study of cancer will also apply to these other conditions.

On the positive side, it should be emphasized that an appreciation of the complexity and multiplicity of genetic events is the first step towards understanding these common and often lethal diseases. Moreover, multiple mutations provide multiple targets for intervention. Indeed, it has been shown that when a single normal gene or chromosomal region is introduced into a cancer cell with multiple mutations, cell growth and or invasion can be dramatically inhibited, at least in the test tube[25]. While it is likely to be some time before we can successfully apply this type of therapy to human cancers *in vivo*, it may eventually be possible to develop drugs that will mimic the effect of normal suppressor genes or interfere with the effect of mutant oncogenes. A highlight of the last decade was the discovery of many of the genes that are responsible for human cancer. The next decade should see the characterization of the biochemical and physiological mechanisms that underlie the action of these genes, facilitating novel approaches to both therapy and prevention.

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# EXHIBIT J

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# Molecular Mechanisms Used by Tumors to Escape Immune Recognition: Immunogenetherapy and the Cell Biology of Major Histocompatibility Complex Class I

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**Summary:** In this article, we explore the hypothesis that tumor cells can escape recognition by $CD8^+$ T cells via deficiencies in antigen processing and presentation. Aspects of the molecular and cellular biology of major histocompatibility complex class I are reviewed. Evidence for histology-specific molecular mechanisms in the antigen-processing and -presentation deficiencies observed in some human and murine tumors is presented. Mechanisms identified include down-regulation of antigen processing, loss of functional $\beta_2$-microglobulin, and deletion of specific $\alpha$-chain alleles. Finally, we discuss studies using an antigen-presentation-deficient mouse tumor as a model for the immunogenetherapy of an antigen-presentation deficiency. **Key Words:** Major histocompatibility complex class I—Tumor—Antigen processing—Antigen presentation—$CD8^+$ T cells—Vaccinia virus, recombinant.

Tumor immunologists once vigorously debated the existence of tumor-associated antigens (TAAs). This debate has generally been resolved, since numerous researchers have shown, in a variety of systems, that tumors can be recognized and, in some cases, destroyed by elements of the immune system (1–4).

The reasons why tumor cells may express TAAs are beginning to be understood. For example, TAAs may be the result of the processes of carcinogenesis, which are generally thought to stem from damage to a large number of genes, some of which have a role in the molecular mechanisms regulating cell growth and division (5). This damage results in the uncontrolled cellular proliferation th defines the transformed cell. Thus, possible origin of TAAs include self-proteins, such as fetal antigen (6), oncogene products (including fusion proteins), mutated tumor-suppressor gene products, other mutated cellular proteins, or such foreign proteins as viral gene products (including but not limited to products of oncogenic viruses). Nonmutated cellular proteins may also be antigenic if they are expressed aberrantly (e.g., in an inappropriate subcellular compartment) or in supranormal quantities (7). Finally, totally normal proteins could be recognized by autoreactive T cells (8).

Given the numerous steps of cellular transformation and the sometimes bizarre genotypes observed in cancer cells, it could be argued that tumor cells are likely to contain many new antigens potentially recognizable by the immune system. Nevertheless every death resulting from progressive cancer ca

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be thought of as resulting from a tumor that has not been eradicated by the immune system. Why is this the case?

## ESCAPE OF TUMOR CELLS FROM IMMUNE RECOGNITION

Escape from immune recognition by tumor cells could be the result of many mechanisms. Considered here are those possible mechanisms involving the target cell (i.e., tumor cell) and the antigens associated with it. Clearly, there could be loss of antigenic epitopes on tumor cells as a result of lack of expression of the entire protein in question. Alternatively, the epitope recognized could mutate to form a structure not recognized because of a "hole" in the repertoire of the immunologically relevant molecules. In some cases, this lack of recognition could be the result of self mimicry, or tolerance. In other cases, lack of recognition of an epitope could be the result of biophysical constraints to recognition. These mechanisms could be operative in the escape of tumors from elements of both the humoral and the cellular immune systems.

Because T cells recognize antigen only after it has been processed and presented by molecules of the major histocompatibility complex (MHC), there is another, entirely different set of occurrences that could play a part in the escape of tumor cells from recognition by T cells (9). An understanding of the molecular structures implicated in recognition of antigens by T cells (reviewed in ref. 10) led to the hypothesis that some tumor cells escape recognition by T cells not because of antigenic loss or mutation but because of a failure to process and present TAAs. Thus, a tumor might evade recognition by T cells by failing to present tumor antigens that were not absent but instead hidden intracellularly and not presented on the cell surface. The second part of this hypothesis is that if the deficiencies in antigen processing and presentation could be corrected, then tumor cells could be immunologically recognized by T cells.

Many investigators have shown that the recognition and destruction of tumor cells by T lymphocytes is due, in part, to the activity of $CD8^+$ T cells ($T_{CD8^+}$). $T_{CD8^+}$ that specifically lyse human and murine tumor cells can be generated in vitro and can eliminate established tumor in vivo (1–4). Antitumor $T_{CD8^+}$ have also been shown to specifically release cytokines when exposed to the appropriate tumor target (11). We chose to study $T_{CD8^+}$ recognition for these reasons.

## THE CELL BIOLOGY OF ANTIGEN PROCESSING AND PRESENTATION

The specificity of T-cell recognition is largely due to the T cell receptor (TCR). The molecular structures of TCR and immunoglobulin (Ig) molecules are very similar and are likely to have evolved from the same primordial molecule (12). However, these two classes of molecules recognize fundamentally different molecular targets. Ig molecules recognize antigen in native or denatured conformations. T cells generally can recognize only processed antigens that are presented by self–MHC molecules (Table 1). MHC molecules presenting peptide antigens designated immunogenic trigger a T-cell response that can consist of proliferation, upregulation of surface molecules, activation of lytic processes, and/or secretion of cytokines.

$T_{CD8^+}$ recognize MHC class I molecules bearing peptides of eight to 10 residues (13) derived from proteins located in the cytosol (14). Association of these peptides with class I $\alpha$ chains and $\beta_2$-microglobulin ($\beta_2$m) is thought to occur in the endoplasmic reticulum (ER) or in an intermediate secretory compartment. Generation of peptide fragments eight to 10 residues in length is likely to require the unfolding of proteins containing antigenic epitopes, but this requirement for unfolding is entirely speculative. Even more speculative is the possible role of members of the heat-shock family of proteins in the unfolding process.

There is clear evidence that antigenic fragments presented by MHC class I molecules are generated by proteolysis, but very little is known about their molecular structures. Ubiquitin-targeted proteolysis may play a part. There is circumstantial evidence that a large nuclear and cytosolic proteolytic structure (termed the proteasome), physically associated with two MHC gene products called LMP-7 and LMP-2 (formerly known as RING 10 and RING 12, respectively), is involved in the production of

TABLE 1. *Toward a molecular understanding of immune recognition of antigen*

| | Humoral | Cellular |
|---|---|---|
| Recognizing cell | B lymphocyte | T lymphocyte |
| Recognizing molecule | Immunoglobulin | T-cell receptor |
| "Self" molecules required? | No | Yes |
| Phase of antigen | Fluid or solid | Solid |
| State of antigen | Native or denatured | "Processed" |

antigenic peptides or their delivery to class I molecules. Two recent reports, however, indicate that the LMP-2 and LMP-7 gene products are not necessary for the presentation of some antigens (15,16). The function of these gene products in antigen processing, therefore, remains to be established.

MHC class I molecules clearly have some role in the generation of properly sized antigens. Falk and colleagues have shown that the peptide fragments recovered from an antigen-presenting cell are dependent on the class I molecules expressed by that cell (17). There are two competing explanations for this phenomenon. The first is that cytoplasmic proteins are broken down to generate extremely short-lived intermediates, and class I molecules "capture" the right fragments, protecting them from further degradation. Alternatively, cytoplasmic proteins could be broken down into longer peptide fragments that are held by class I molecules while cellular proteases trim them to the appropriate lengths. This could be called the "cookie cutter" hypothesis. Elements of both of these hypotheses may play a role in fitting the peptide pool to the available class I.

How are antigens transported into the ER? A great deal of evidence suggests that peptide antigen associates with class I and $\beta_2$m in the ER or in some post-ER compartment (10). Two gene products that are clearly involved, designated TAP-1 and TAP-2 (for *t*ransporter associated with *a*ntigen *p*rocessing), are encoded in the MHC. Based on sequence homology, these genes are members of the ABC transporter family. TAP-1 (previously known as RING 4, Y3, and PSF-1) and TAP-2 (previously known as RING 11, Y1, and PSF-2) are clearly needed for cells to efficiently process antigen (reviewed in ref. 18). Based on size and predicted structure, it seems likely that these proteins directly transport peptide from the cytosol, but this theory is unproved.

One member of the heat-shock protein family of proteins (HSPs), called gp96, has been proposed by Srivastava (unpublished observations) to be capable of binding peptides that could subsequently be presented by class I molecules (19). These HSPs may help stabilize unfolded, class I heavy-chain molecules and then transfer antigenic peptides to these molecules, leading to the formation of the stable trimolecular complex of class I α-chain, $\beta_2$m, and peptide antigen. The genes coding for class I α-chains, the putative peptide transporters, and the proteasome component molecules appear to be very closely associated with the MHC regio chromosome 17 in the mouse, or chromosome the human (for map, see ref. 20). Each of t genes, along with $\beta_2$m and gp96, is inducible interferon-γ (IFN-γ).

## ANTIGEN-PROCESSING AND -PRESENTATION DEFICIENCIES IN HUMAN CANCERS

Several molecular mechanisms by which tu cells fail to process and present endogenous a gens to $T_{CD8}$- could be hypothesized (Table 2). ing isolates of tumor cells from patients at the tional Institutes of Health to establish cell lines in vitro studies and fresh tumor sections for im nohistochemical studies and in vivo correlates have found that at least three mechanisms are erative. The first mechanism, identified in five m anoma cell lines (N. P. Restifo, unpublished ob vations), involves the loss of functional $\beta_2$m exp sion, similar to that seen by D'Urso et al. (21). T antigen-presentation deficit documented in th cell line studies is entirely correctable by gene sertion of $\beta_2$m. A second mechanism, also obser in melanoma, is the loss of expression of partic MHC class I alleles (F. Marincola, unpublished servations), and it, too, is correctable by inserti of the missing α-chain genes. The third type of fect, observed originally in small-cell lung can histology, stems from the down-regulation of c tain molecules that putatively process antigens, cluding TAP and MHC-encoded proteasome co ponents (22). This mechanism is correctable b treatment with exogenous IFN-γ or by insertion the IFN-γ gene. It is this final mechanism that wi be discussed in detail below.

In our attempts to examine the capacity of huma tumor cell lines to process cytosolic antigens fo $T_{CD8}$- recognition, we were faced with the proble

TABLE 2. *Mechanisms of escape of human tumor cells from recognition by $T_{CD8}$- relating to antigen processing and presentation*

| Gene lost, mutated, or Underexpressed | Result | Cell-surface class I (MAb W6/32) |
|---|---|---|
| TAP 1, 2 (LMPs, proteases?) | Poor processing of peptide antigens | Decreased |
| $\beta_2$-Microglobulin | α-Chain instability | Absent |
| α-Chain alleles | No presentation | Absent–norm |

MAb, monoclonal antibody; LMP, low-molecular-weight pr teins.

of the massive polymorphism of the MHC. Since class I molecules are among the most polymorphic genes in the human genome, we were able to consistently use one class I molecule, to present one antigenic epitope for recognition by one population of effector cells, to study the antigen-processing system. Instead, early studies focused on the cumbersome task of tailoring reagents for particular MHC alleles. We thus devised a method for screening a large number of tumor-cell isolates that was independent of both the human leukocyte antigen type of the tumor and the presence or absence of specific cellular proteins. This method exploits the capacity of recombinant vaccinia virus (rVV) to infect a wide variety of human tumor cells. Using an rVV encoding the mouse H-2 $K^d$ class I molecule ($K^d$-VV), we could test human tumor cell lines for presentation of viral antigens to mouse $K^d$-restricted, VV-specific $T_{CD8^+}$ populations and thus study the antigen-processing capabilities of human tumor cells per se.

One of the earliest observations made had nothing to do with the processing of intracellular antigens. Instead, five melanoma cell lines (of ~60) were found deficient in the expression of functional $\beta_2$m (N. Restifo, unpublished observations). As mentioned above, supplying an intact $\beta_2$m gene using an rVV completely restored the antigen-presentation function of these melanomas.

In another set of experiments (22), 26 tumor cell lines were infected with $K^d$-VV and tested for lysis by VV-specific $T_{CD8^+}$. In each experiment, the T2 cell line was included as a negative control. T2 cells lack a 1-megabase region of the MHC that contains the portion coding for the TAP genes and proteasome component molecules and are known to be deficient in their capacity to process viral antigens for $T_{CD8^+}$ recognition (23,24). These experiments showed that human tumor cell lines vary widely in their capacities to process and present viral antigens to $T_{CD8^+}$. While some cell lines with a variety of tumor histologies were lysed at high levels by VV-specific cytotoxic T lymphocytes (CTLs), many were lysed at low levels. Notably, all six of the small-cell lung carcinomas (SCLCs) studied were consistently recognized at levels similar to or even lower than T2 cells. Figure 1 shows an example of the kinds of functional data obtained from these experiments.

The failure of VV-specific $T_{CD8^+}$ to lyse various tumor cells could not be attributed to low levels of expression of VV gene products. SCLCs generally expressed high levels of viral antigens relative to

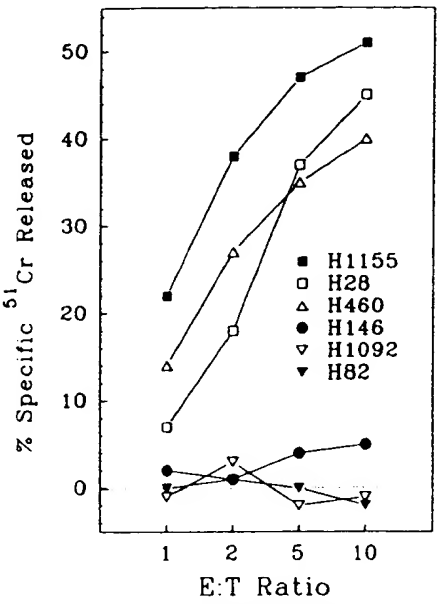


FIG. 1. Screening of human tumor lines for antigen-processing defects. Cell lines studied were cultured human tumor cells tested for their capacity to present vaccina virus (VV) antigens to $K^d$-restricted, VV-specific $T_{CD8^+}$. The tumor cell lines were provided by J. Minna (University of Texas, Southwestern). H1155, H28, and H460 are all non-small-cell lung cancers. H146, H1092, and H82 are all small-cell lung cancers. In the representative experiment shown, all tumor cell lines processed and presented endogenous antigens poorly.

the other tumor cell lines studied. The failure of $T_{CD8^+}$ to lyse VV-infected SCLCs appeared to be due to a genuine defect in antigen presentation and not to other factors.

Pulse-chase methodology was used to study the biosynthesis and intracellular transport of class I molecules (22). These studies confirmed that class I molecules remained completely sensitive to digestion with endo H throughout an 80-min chase period. Thus, class I molecules were not transported through the Golgi complex to the cell surface. IFN-$\gamma$ treatment of SCLC cells greatly altered the intracellular trafficking of class I molecules, inducing the transport of a substantial portion of $K^d$ molecules from the early portion of the secretory pathway through the Golgi complex. This was shown to be the case, since approximately half of the class I molecules immunoprecipitated became resistant to endo H digestion within 80 min of their synthesis. The acceleration of the intracellular transport of class I molecules was associated with higher steady-state class I expression on the cell surface, similar to that seen in the murine tumor system described below (25).

Retention of class I molecules in an endo H–sen-

sitive form has been observed in cells that fail to express TAP or MHC–proteasome subunit gene products (26). When the RNA from the SCLC lines was probed for the expression of message from the TAP and MHC–proteasome subunit molecules, no mRNA could be detected. Low or absent steady-state levels of mRNA indicated either that transcription was down-regulated or that there was a shortening of the half-life of these messages. Our inability to detect these relevant mRNAs by Northern blot analysis did not indicate a deletion of the genes in these cells, since treatment of cells with IFN-γ for 48 h induced the expression of large quantities of mRNA encoding the four genes. Perhaps most importantly, IFN-γ greatly enhanced the antigen-presenting capacity of the SCLCs, but not T2 cells, in functional assays of antigen presentation (22). IFN-γ had no discernible effect on the presentation of peptide that was exogenously provided, despite the fact that it was clearly capable of enhancing presentation of VV antigens to VV-specific $T_{CD8^+}$.

Our studies with SCLC cell lines did not address the question of whether poor antigen processing was associated with transformation or outgrowth of the tumor or whether this poor processing was representative of the natural regulation of class I expression in the tissue of origin. It is, however, unlikely that the poor antigen-processing capacities exhibited by SCLC cells is an artifact resulting from cell culture, since immunohistochemical studies show that SCLCs express very low or undetectable levels of class I in vivo (27). Thus, poor expression of class I molecules on the cell surface could be associated with a profound incapacity to process endogenously synthesized proteins.

## DEVELOPMENT OF A MOUSE MODEL

The great enhancement by antigen processing of IFN-γ treatment of SCLCs suggested that recognition of tumor cells by anti-tumor $T_{CD8^+}$ could be enhanced by the specific up-regulation of the antigen-processing system in tumor cells. Antigen processing and presentation by tumor cells was conceivably a limiting step in the activation of antitumor $T_{CD8^+}$, especially in tumors expressing low levels of class I. In order to test a therapeutic intervention in animals, we developed a mouse model.

Of two large panels of methylcholanthrene-(MCA) induced sarcomas generated in our laboratory, seven have been extensively characterized (28,29) (summarized in Table 3). An eighth tumor,

TABLE 3. *Summary in vitro and in vivo characteristics of MCA-induced sarcomas*

| Tumor | Anti-tumor CTLs in vitro | Immunogen in vivo | Relati MHC cl |
|---|---|---|---|
| MC 38 | Yes | Yes | 423 ± |
| MCA 207 | Yes | Yes | 249 ± |
| MCA 205 | Yes | Yes | 110 ± |
| MCA 203 | Yes | Yes | 64 ± |
| MCA 105 | Yes | Yes | 32 ± |
| MCA 102 | Yes | No | 28 ± |
| MCA 101 | No | No | 7 ± |
| MCA 106 | Yes | Yes | 4 ± |

MC 38, a murine colon adenocarcinoma, has a been characterized. Of these tumors, MC 38, M 105, 203, 205, and 207 were described as immu genic since after simple immunization of tumor *C. parvum*, they protected against subsequent ch lenge with the same tumor. MCA 102 had an int mediate phenotype; it induced a CTL response b did not protect against subsequent challenge. M 106 was found to express low levels of class I b nevertheless, was found to be immunogenic. T tumors studied differed widely in their expressi of class I molecules on the cell surface (29). At o posite ends of the distribution were MC 38 a MCA 101 or MCA 106, which had a 100-fold diff ence in steady-state levels of cell-surface class Similar results were obtained using either fresh t mor explants or cells that had been maintained tissue culture. MHC class II molecules were n detected on the surfaces of any of these tumors b FACS analysis. Thus, MCA 101 expressed very lo levels of class I and neither induced CTLs nor pr tected against tumor challenge.

In studies using model viral systems, in whi tumor cell lines were infected with influenza A/P 8/34 (PR8) virus, MCA 101 was found to be near incapable of presenting endogenous antigens to a tiviral CTLs, despite the fact that MCA 101 w extremely well infected. The antigen-processing c pability of MCA 101 was found to be as poor RMA-S, which was included as a negative contro Several of the other tumors tested also presente endogenously generated viral antigens poorl Since $CD8^+$ CTLs have a proven role in the im mune response against MCA-induced tumors, was not surprising that many of the tumors teste had a lowered capacity to present endogenousl generated viral antigens. In fact, one would expe that tumor cells with antigen-processing or -prese tation defects would enjoy a selective advantage. should be noted, however, that decreased class

expression can make tumor lines more susceptible to lysis by natural killer (NK) cells in some cases (30). Thus, some of the mechanisms used by tumors to escape recognition by CTLs may increase their susceptibility to other cells in the immune system.

To examine the capability of MCA 101 to present individual influenza virus proteins to CTLs, we infected cells with VV recombinants expressing individual influenza A virus proteins that are known to be recognized by H-$2^b$ restricted CTLs (31). This test showed that while nucleoprotein (VV-NP), nonstructural 1, hemagglutinin, and acidic polymerase were presented by MCA 207 (an immunogenic tumor), none of the proteins were presented by MCA 101 over control levels obtained with a VV recombinant expressing the nucleocapsid protein of vesicular stomatitis virus. These findings indicated that MCA 101 was incapable of presenting a wide variety of proteins to CTLs via the endogenous route.

Our findings show that the nonimmunogenic murine sarcoma MCA 101 presented endogenously, generated influenza virus antigens poorly to CTLs. It should be noted, however, that it remained unclear from these studies whether a complete lack of presentation occurred or whether the number of MHC-peptide epitopes was insufficient to be detected by our polyclonal responder populations. The latter explanation seems more likely. It is important to note that as with the SCLC cell lines, the presentation of endogenous antigens by MCA 101 was made possible by up-regulating class I expression with IFN-γ.

## AN IMMUNOGENETHERAPY DESIGNED TO CORRECT DEFICIENT ANTIGEN PROCESSING

The nonimmunogenic murine MCA-induced sarcoma MCA 101 had a very poor capacity to present endogenously generated viral antigens. It grew rapidly and lethally in nonimmunosuppressed mice. Furthermore, of seven MCA-induced tumors generated and characterized extensively in our laboratory, only MCA 101 could neither act as an immunogen in vivo nor generate CTLs in vitro (Table 3).

Our goal in the next series of experiments (25) was to genetically modify MCA 101 to convert it into a good presenter of endogenous antigens. We thought that this modification could be accomplished by transducing the cells with the cDNA for murine IFN-γ. Thus, our aim with the gene-insertion studies was not to use IFN-γ for its direct effects on cells of immune lineage, but instead for its effects on the antigen-presentation capabilities of the tumor cells before and after gene modification. We then hoped to correlate the in vitro antigen-processing capabilities with in vivo behavior of these cells as immunogens.

Using this system, it was thus possible to answer the question of whether MCA 101 was nonimmunogenic because it lacked TAAs or because of its failure to present some postulated, but as yet unidentified, TAA. Since the amelioration of the capacity of MCA 101 to present endogenous antigens after treatment in vitro with exogenous mIFN-γ was transient and correlated with the duration of class I expression, we hypothesized that retroviral transduction of MCA 101 with the cDNA for mIFN-γ would have the effect of prolonging the processing and presentation of endogenous antigen by virtue of the stable insertion of the gene.

IFN-γ-transduced MCA 101 expressed much higher levels of surface class I molecules, as measured by FACS analysis, then the neomycin resistance gene ($Neo^R$) bulk-transduced cells and wild-type MCA 101. This pattern of very low or unmeasurable IFN-γ production with greatly increased class I production was similar to that seen by Gansbacher and colleagues (32). Increased class I expression was seen in both fresh and cultured tumor lines.

In an effort to test the effect of IFN-γ gene transduction on the capability of MCA 101 to present endogenous antigens, we infected wild-type MCA 101, IFN-γ gene-modified MCA 101, and MCA 101 tumor gene modified with the neomycin resistance gene alone with VV genetically engineered to express influenza A genes (25). Effector cells were splenocytes from B6 mice stimulated in vivo with VV-NP and in vitro with the NP peptide. Wild-type MCA 101 was not killed when it was sham-infected, when it was infected with the control VV containing the neuraminidase gene, or when it was infected with the VV engineered to express the influenza A nucleoprotein gene (VV-NP). IFN-γ gene-modified tumor cells were also not killed when sham-infected or when infected with a control virus, but they were specifically killed when they were infected with the VV-NP virus. Like wild-type MCA 101, MCA 101 modified with the neomycin resistance gene alone did not present viral antigens, indicating that the improvement in the capability of IFN-γ gene-modified MCA 101 to present antigen was not simply due to the effects of retroviral transduction. It

thus appeared that by inserting the mIFN-γ gene into a bulk population of wild-type MCA 101, we could convert it from a poor presenter of antigen to a cell line capable of presenting antigen to a similar extent as our most immunogenic tumors (25). This finding led us to hypothesize that MCA 101 might be capable of presenting its own tumor antigens in vivo.

## EFFECTS OF IFN-γ GENE TRANSDUCTION ON THE IMMUNOGENICITY OF MCA 101

Generation of successful cultures of tumor-infiltrating lymphocytes (TILs) from wild-type MCA 101 had not been achieved in our laboratory. In order to obtain pure cultures of uniformly high class I–expressing transductants, the bulk-transfected MCA 101 (101.NAT) was cloned. We found that the insertion of the cDNA for IFN-γ caused increased expression of steady-state surface class I, as measured by FACS analysis, on some but not all of the tumor clones tested. Despite their variable levels of class I expression, none of the clones secreted >5 U of mIFN-γ/$10^6$ cells/ml/24 h by ELISA. When clones were implanted subcutaneously into syngeneic B6 mice, no significant differences were found between the growth rates of high class I–expressing and low class I–expressing tumors in vivo.

Antitumor T cells could be grown only from high class I–expressing transductants (25). This was done by harvesting subcutaneous tumors after 10–20 days, then immunobeading single-cell suspensions of tumor digests using immunomagnetic beads coated with Thy $1.2^+$ antibodies. Like other murine TILs generated in our laboratory, the TIL cultures used in vivo were found to be exclusively $CD8^+$ by FACS. These antitumor CTLs were then expanded in vitro in 20 U/ml of rhIL-2 and tested in vivo and in vitro.

In an effort to test whether the TILs generated from high class I–expressing mIFN-γ-transduced clones could be active in an adoptive immunotherapy model against established wild-type tumor in vivo, we used the 3-day lung metastases model. In these experiments, mice were injected intravenously with fresh wild-type MCA 101 or IFN-γ gene-modified MCA 101 tumor cells. On day 3, mice were treated with saline alone, interleukin-2 (IL-2) (10,000 U) in saline twice daily for 5 days, or the same dosage of IL-2 plus varying dosages of TILs. Mice were killed on day 14, when their lungs were harvested and counted in a blinded fashion the number of pulmonary tumor nodules. Most nificantly, TILs generated from the mIFN-γ ge modified MCA 101 tumor clones expressing h levels of class I were effective against pulmon metastases from the wild-type MCA 101 tumor.

TILs generated from IFN-γ gene-modified tu were tested for release of cytokines after coincu tion with appropriate tumor cells. TILs deri from high class I–expressing, IFN-γ gene-modif tumor clones were tested by exposure to a vari of stimuli. Maximal release was gauged when TI were stimulated with an anti-CD3 antibody, 2C When TILs were incubated alone, without stimu tion, background levels of tumor necrosis facto (TNF-α) and IFN-γ were relatively low for b cytokines. Fresh tumor preparations alone w found to secrete little, if any, IFN-γ. Production TNF was variable by fresh tumor preparations was uniformly <20 U/$10^6$ cells/ml/24 h. Values tained from tumor alone were subtracted from ues obtained from tumor plus TILs. The most portant finding was that wild-type MCA 101 stim lated release of IFN-γ and TNF-α almost as well IFN-γ gene-modified MCA 101. Thus, TILs gen ated from mIFN-γ, gene-modified tumor clon could be triggered to secrete, in a relatively tum specific fashion, IFN-γ, and TNF-α not on against high class I–expressing tumor clones b also against the low class I–expressing wild-ty tumor.

The MCA 101 tumor was not consistently lys by therapeutic TILs. However, it did stimulate t release of cytokines with relative specificity. O possible explanation of this effect is that the me surement of cytokine release is more sensitive tha assays of cytotoxicity. This finding agreed with pr vious data, which showed that $CD8^+$ TILs th were therapeutic in vivo were not in every ca cytotoxic (11).

Increased antigen density on mIFN-γ gen modified MCA may explain why a therapeutical useful $CD8^+$ T-cell response can be obtained fro high class I–expressing clones and not from lo class I–expressing mIFN-γ-transduced clones, co trol $Neo^R$ transduced clones, or wild-type tumo There is evidence in tumor immunology studies th an immunologically strong afferent tumor stimul can elicit a response against an immunological much weaker efferent stimulus (33).

While IFN-γ-transduced tumor lines and clon secreted little, if any, IFN-γ into culture superna

tant, the effect of the cytokine could act primarily via intracellular IFN-γ receptors. We have inferred that the observed effects of IFN-γ transduction are due to the enhancement of the transduction of certain genes involved in the processing and presentation of endogenous antigen (25). However, IFN-γ has many effects and may be enhancing the outgrowth of antitumor T cells through an entirely different mechanism. For example, IFN-γ has been shown to be a potent up-regulator of certain accessory molecules, ICAM-1 among them, and while it was not specifically addressed in the studies presented here, the effect of IFN-γ on these molecules may play an important role in the phenomena described here. Another possibility is that IFN-γ acts primarily through up-regulation of class II expression on macrophages and dendritic cells, thereby enhancing antigen presentation to T-helper (Th) cells. IFN-γ may alter the responsiveness of other immune cells. For example, the observed effects of tumor transduction with the gene for IFN-γ may be due partly or entirely to the enhancement of Th1 responses with a concomitant diminution of Th2 responses. This conversion has been shown to occur under the influence of IFN-γ in other systems (34). The relative contributions of these mechanisms are currently being addressed in our laboratory.

## CURRENT WORK AND FUTURE DIRECTIONS

Genetic or pharmaceutical therapies directed at the enhancement of antigen processing may exploit the beneficial effects of IFN-γ. However, IFN-γ has been shown in some cases to be antiproliferative to T cells and NK cells, and other ways of enhancing antigen processing and presentation might prove to be more therapeutically useful. It seems likely that the genes involved in antigen processing and presentation share common regulatory elements. When these regulatory elements are elucidated, new therapies could be developed for specific up-regulation of antigen processing in cancer and infectious disease. Conversely, down-regulation of these gene products may prove to be useful in tissue transplantation or in autoimmune disease.

Other investigators have recently shown the antimetastatic effects of vaccination of tumor-bearing mice by immunization with IFN-γ gene-modified tumor cells (35). Furthermore, the use of tumor cells gene modified with the cDNA for IFN-γ has been extended, and partially confirmed, in our own laboratory by Shiloni and colleagues (unpublished observations). In these studies, one high and one low class I–expressing clone of the nonimmunogenic MCA-102 fibrosarcoma, designated 4JK and 24JK respectively were retrovirally transduced with the cDNA encoding for murine IFN-γ. Retroviral transduction of tumor cells with the cDNA encoding for IFN-γ resulted in a substantial up-regulation of class I surface expression in the 24JK clone but little change in the 4JK clone. In an attempt to generate antitumor lymphocytes, these gene-modified cells were inoculated into mouse footpads, and draining lymph nodes (DLNs) were removed, dispersed, and cultured in vitro for 10 days with irradiated tumor cells and IL-2. DLNs from mice bearing either unmodified tumor or tumor transduced with cDNA encoding for $Neo^R$ or IFN-γ were used to treat recipients harboring 3-day pulmonary metastases induced by the parental unmodified tumor. Treatment with DLN cells obtained after the injection of 24JK tumor cells modified with the gene for IFN-γ significantly reduced the number of pulmonary metastases in four separate experiments, compared with groups treated by DLN cells generated from inoculation of either the unmodified, parental 24JK clone or the same clone transduced with the $Neo^R$ gene only. In contrast, DLN cells induced by either IFN-γ-transduced 4JK (high MHC class I expressing) tumor or an unmodified 4JK tumor (moderate MHC class I expressing) had significant but equal therapeutic efficacy. Although the in vitro growth rate of tumor cell lines was unaffected by the insertion of the mouse IFN-γ cDNA, their in vivo (s.c.) growth rates were significantly slower than those of the nontransduced tumors. Thus, after retroviral transduction of the murine IFN-γ cDNA into a nonimmunogenic tumor with a very low level of surface expression of MHC class I, modified tumor cells could elicit therapeutic T cells from DLNs capable of successfully treating established pulmonary metastases upon adoptive transfer. This strategy confirms previous observations on the potential therapeutic effects of gene modification of tumor cells with IFN-γ. Significantly, these studies further define the circumstances in which IFN-γ immunogenetherapy might be useful.

In conclusion, new immunogenetherapeutic strategies may be based on a more detailed understanding of the molecular mechanisms used by tumors to escape immune recognition. A more complete knowledge of the cell biology of MHC class I in health and disease can lead to new strategies aimed at enhancing processing and presentation of tumor

antigens. These approaches may lead to the generation of immune responses against tumor histologies not previously thought to be susceptible to T-cell based immunotherapies of cancer.

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